Preparation of *3-D Life* Hyaluronic Acid (HA) Hydrogels

1. Introductory Notes

- *3-D Life* Hyaluronic Acid Hydrogels are biochemically defined hydrogels that can be applied for three-dimensional cultivation of many cell types.
- Ease of use and complete control of bio-molecular modifications and gel stiffness allow an extensive variety of cell culture applications.
- The hydrogel is formed by the cross-linking of thiol-reactive dextran or polyvinyl alcohol (PVA) with thiol-functionalized hyaluronic acid. The presence of hyaluronic acid allows the spreading and migration of most cells, if cell adhesion molecules (for example RGD Peptide, Cat. No. 09-P-001) are present in the gel.
- If combined with a thiol-reactive dextran, *3-D Life* Hyaluronic Acid Hydrogel can be dissolved by the addition of *3-D Life* Dextranase (Cat. No. D10-1) to recover chemically fixed or live cells.

2. Protocol

The following protocol describes the preparation of *3-D Life* Hyaluronic Acid Hydrogels for 3-D cell culture with and without modification of the gel matrix 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 PVA-HA Hydrogel (Catalog Number G85-1) **or**

3-D Life Dextran-HA Hydrogel (Catalog Number G95-1)

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.

Preparations

Hydrogel reagents:

- Dissolve the HyLink lyophilisate of the *3-D Life* PVA-HA or *3-D Life* Dextran-HA Hydrogel kits according to the instructions in the accompanying 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, put the buffer vial in a 37°C water bath until all salts are dissolved. Do not put 10x CB (pH 7.2) on ice, this may cause the

salts to crystallize.

- If RGD Peptide is used, reconstitute the RGD Peptide according to the instructions on the accompanying Product Data Sheet.

Note: Do not expose thiol-containing reagents (RGD Peptide, HyLink) 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 (cross-linking strength of 1.2 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.

Table 1: Reagent volumes for 100 µl of gel using SG-Dextran or SG-PVA polymer to be cross-linked with 1.2 mmol/L SH groups of the cross-linker HyLink (1.2 mmol/L cross-linking strength) with the option of modification with 0.5 mmol/L RGD Peptide.

Reagents	Volumes for 100 µl gel	
	w/o RGD Peptide	with RGD Peptide
Water	56.0 µl	51.8 µl
10x CB, pH 7.2	8.0 µl	8.0 µl
SG-Dextran or SG-PVA (30 mmol/L SH-reactive groups)	4.0 µl	5.7 µl
RGD Peptide (20 mmol/L SH groups)	0.0 µl	2.5 µl
Cell suspension	20.0 µl	20.0 µl
HyLink (10 mmol/L SH groups)	12.0 µl	12.0 µl
Total	100.0 µl	100.0 µl

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 homogeneous 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 cross linker HyLink. Mix by pipetting up and down a few times.
5. After addition of the cross linker incubate the pre-gel solution for up to 6 minutes (SG-PVA) or 8 minutes (SG-Dextran) at room temperature. Do not incubate longer because the solution will

begin to solidify and will not be pipetable 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 25 minutes at room temperature or at 37°C in the incubator to allow the gel to solidify.

6. Make sure that the gel has solidified and 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. The volume of 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-Peptidee, 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 cross linker 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 cross linker and the beginning of solidification of the gel solution becomes considerably shorter. In Table 2 approximate 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 cross-linking strengths of 1 to 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 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 hydrogels crosslinked with HyLink at different grades of stiffness (cross-linking strength) at room temperature.

Crosslinking strength	Time after mixing polymer and cross linker			
	SG-Dextran + HyLink		SG-PVA + HyLink	
	Fluid up to	Addition of medium after	Fluid up to	Addition of medium after
1 mmol/L	15 min	30 min	10 min	30 min
1.2 mmol/L	8 min	20 min	6 min	25 min
1.4 mmol/L	3 min	17 min	2.5 min	15 min
2 mmol/L	2 min	8 min	1 min	8 min

Slowing down gelation by pH reduction for highly crosslinked hydrogels

If hydrogels of higher stiffness than indicated in Table 2 are prepared, the time to keep the pre-gel solution fluid and pipettable can be extended by reducing the pH. Usually, the preparation of *3-D Life* Hyaluronic Acid Hydrogels is performed at pH 7.2. 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). Such a mixture of 10x CB can be used instead of 10xCB (pH 7.2) to slow down the gel formation.

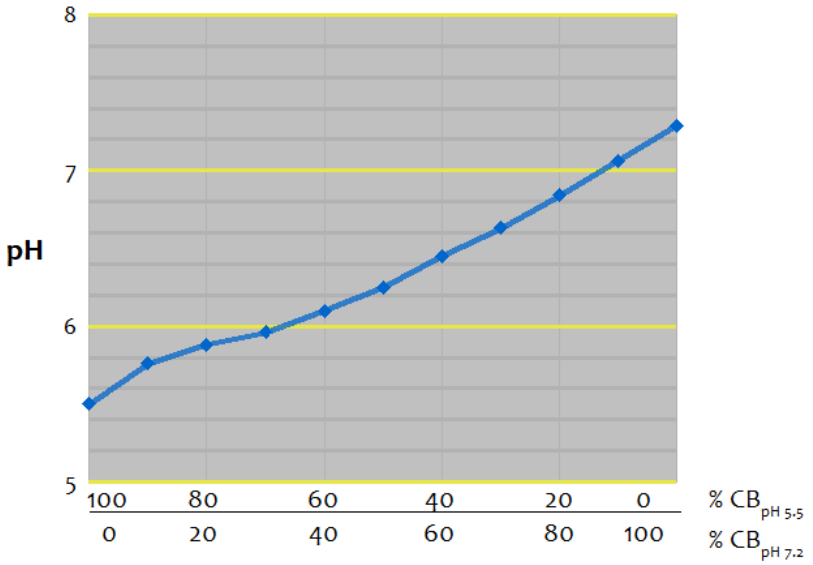

Fig. 1: pH resulting from different mixing ratios of 10x CB $_{pH5.5}$ and 10x CB $_{pH\ 7.2}$.

4. Dissolving SG-Dextran Hydrogels with Dextranase

Live or chemically fixed cells can be recovered from Dextran-HyLink 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 remnants of dextranase and dissolved gel components. The removal of dextranase is important when cells are being embedded again in dextran hydrogels for a continued culture. Small traces of dextranase can destabilize the newly set up hydrogel.