Application Note

Creating Sodium Alginate beads directly in ClinoReactor using a Triple-Negative Breast Cancer (TNBC) cell line

- This application note details a protocol using sodium alginate to create 3D constructs for culturing in the ClinoStar system.
- Sodium alginate embedding provides a scaffold and protection of cells, which facilitates dynamic 3D culture without clumping.
- Sodium alginate can be used as a reproducible and easy gel embedding technique.
- Using colored sodium alginate increase constructs visibility and all-over ease of use.

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Introduction

Sodium alginate embedding provides a means to create 3D cell conglomerates [1]. It is particularly useful for production of constructs of cells which are challenging in single cell suspension or pre-aggregation setup [2]. Constructs created using this protocol, are of similar size and contain similar starting cell number. These constructs provide a structural microenvironment for minimum 28 days, preserve cell viability, and allow cell growth. Constructs made using colored sodium alginate have increased visibility for easier handling.

It is important to note that during all preparation steps, solutions without calcium and magnesium ions should be used, as those ions otherwise will trigger sodium alginate polymerization.

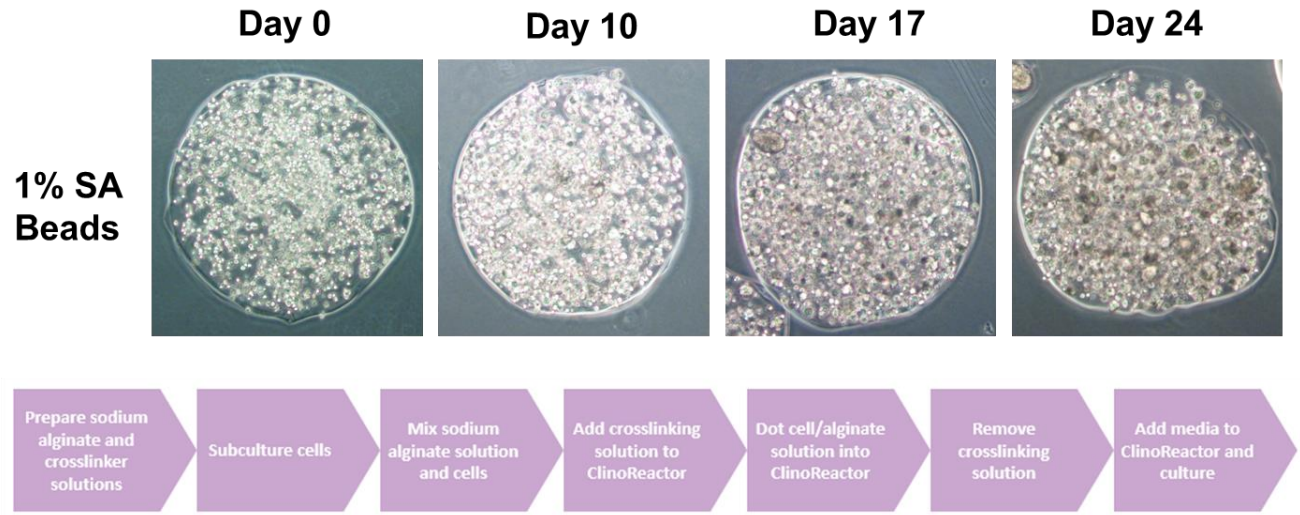


FIGURE 1 Bright field images of 1% sodium alginate (SA) beads at different days during culture in the ClinoStar system. Below, a flowchart of the sodium alginate embedding protocol.

Reagents and Materials

- Triple-Negative Breast Cancer (TNBC) cell line MDA-MB-231 cells (HTB-26, ATCC)
- Growth media
- Hanks' Balanced Salt Solution (HBSS) without calcium and magnesium (14175053, Gibco)
- Sodium alginate (W201502, MERCK)
- Cross-linking solution (150 mM NaCl and 50 mM Ca_2Cl in miliQ water)
- Erioglaucine disodium salt (E133) (861146-5G, Sigma-Aldrich)
- Sterile filter, 0.22µm (514-1259, VWR)
- ClinoReactor®
- ClinoStar®
- Single channel electronic pipette in multiple dispensing mode for 1 µl. (Biohit, E10)
- Gel loading tip (F1731281, Pipetman)
- Syringe (B. Braun, 4616103)
- Needle (B. Braun, 466512)
- Micro BCA™ Protein Assay Kit (23235, ThermoFisher)
- CellTiter-Glo® Luminescent Cell Viability Assay (G7570, Promega)

Protocol

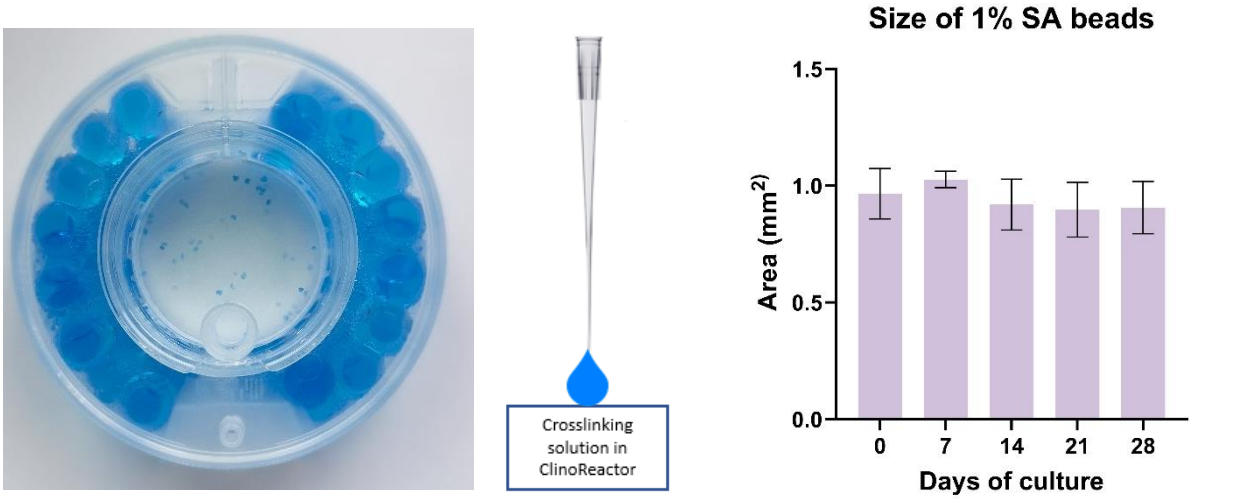


FIGURE 2 Left: Blue sodium alginate (SA) beads are formed directly into the ClinoReactor. ClinoReactor contains 60 sodium alginate beads colored with E133. The gel tip shows how alginate/cell suspension should be dotted into crosslinking solution. Right: Column chart displaying the size of 1% sodium alginate (SA) beads at different days during culture in the ClinoStar system (n =3 in each group).

Preparation

1. Solubilize sodium alginate in HBSS without calcium and magnesium, creating a **1%** sodium alginate solution.
2. Sodium alginate is solubilized by stirring and heating to 70°C on a heating plate (approximately one hour).
3. Dip a p200 pipette tip into the E133 powder dye and add to sodium alginate solution (at room temperature). (NB: it is possible to omit color, see note 2 in comments).
4. Prepare isotonic cross-linking solution containing 150 mM NaCl and 50 mM $CaCl_2$ in H_2O (MiliQ water).
5. Sterilize sodium alginate solution and cross-linking solution by filter sterilization (0.22µm (514-1259, VWR).
6. Hydrate and equilibrate ClinoReactors.
7. Sub-culture and count MDA-MB-231 according to ATCC protocol but resuspend cells after centrifugation in HBSS without calcium and magnesium.

Preparation of alginate/cell suspension

1. Transfer 2 million cells to a 15 mL falcon tube.
2. Add 10 mL of HBSS to dilute the presence of Ca^{2+} and Mg^{2+} ions.
3. Centrifuge falcon tube at 130 RFC for 5 minutes.
4. Aspirate supernatant and add 2 mL 37°C sodium alginate and mix well (pipetting up and down ~10 times) to create 2 mL of dyed sodium alginate solution with 1000 cells/µL.
5. Divide sodium alginate/cell solution into aliquots corresponding to number of ClinoReactors to be created (usually with 60 to 100 droplets per ClinoReactor). We recommend no more than three aliquots as the solution auto-polymerize over time.

Dotting of alginate into ClinoReactor

1. Add 5 mL of crosslinking solution to ClinoReactor and position it in petri dish mode (open the front lid).
2. Adjust single channel electronic pipette in multiple dispensing mode to 1 µL with a total of 10 µL.
3. Dot alginate/cell solution into ClinoReactor. Use a gel loading tip to make a drop at the tip (see **Figure 2**) and touch carefully the surface of the crosslinking solution.
4. Discard tip.
5. Repeat step 3 and 4 until 60-100 alginate/cell beads are created.
6. Allow polymerization for 10 minutes after last dot. Allowing the sodium alginate/cell constructs to round up further.

Adding media and future culture

1. Swirl the ClinoReactor to force dots to submerge in the crosslinking solution.
2. Remove any dots with irregular size.
3. Close the lid of the ClinoReactor and position it in an upright position.
4. Remove the crosslinking solution.
5. Fill the ClinoReactor with growth media.
6. Place ClinoReactor in ClinoStar starting at approximately 17 rpm and adjust speed accordingly.

Results

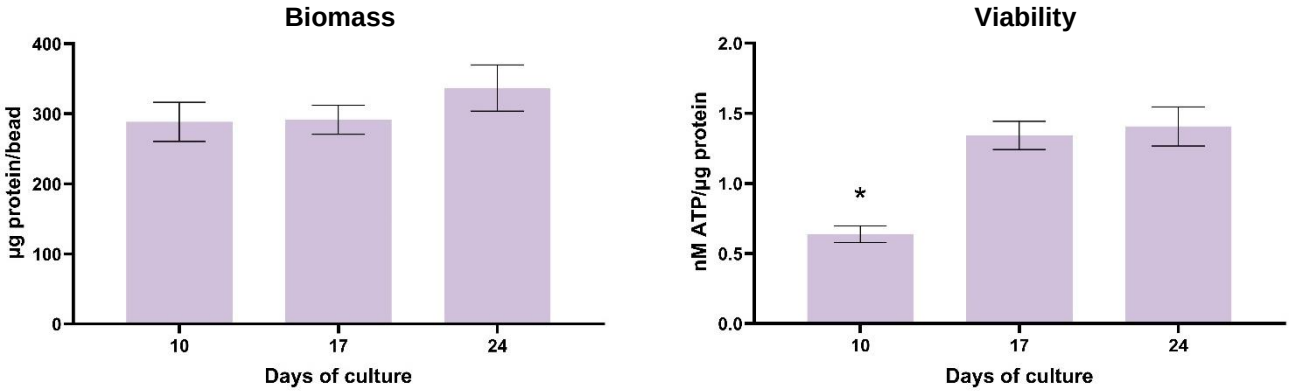


FIGURE 3 Left: column chart displaying biomass as protein content pr. bead for selected time points (n=3 in each group, *p>0.05, One-way ANOVA). Right: column chart displaying viability as ATP content normalized to protein content (n=3 in each group, *p>0.05, One-way ANOVA)

Conclusion

The triple-negative breast cancer (TNBC) cell line, MDA-MB-231, represent a highly aggressive, invasive, and poorly differentiated breast cancer type. MDA-MB-231 lacks oestrogen receptor (ER), progesterone receptor (PR) expression, and HER2 (human epidermal growth factor receptor 2) amplification [3],[4].

It has proven difficult to culture the MDA-MB-231 cell line as single cells suspension and as pre-aggregates using a microwell plate due to extensive clumping in the ClinoStar system, hence the protocol at hand was developed to form individual 3D constructs.

In **Figure 2** it is shown that the beads does not change size over the culture period of 24 days. In **Figure 3** it is shown that biomass as protein content does not increase significantly during the culture period of 24 days, although an increase tendency from day 17 to 24 was observed. Furthermore, it is shown that viability expressed as ATP levels are not decreasing over time, indicating that sodium alginate embedding does not hamper ATP production, hence preserve cell viability.

Comments

1) Sodium alginate embedding can be used for various cell types. For some cells sodium alginate concentration needs to be optimized. For example, MDA-MB-231 cells work best in a 1% solution while HEPG2/C3A cells work best in a 2% sodium alginate solution. In general, we observe that sodium alginate concentration would range from 0.5% to 2%.

2) This step allows to create colored constructs, easy to maintain during culture period. This step could be omitted if one would like to have transparent constructs

References

[1] K. Wrzesinski, H. S. Frandsen, C. Calitz, C. Gouws, B. Korzeniowska, and S. J. Fey, “Clinostat 3D Cell Culture: Protocols for the Preparation and Functional Analysis of Highly Reproducible, Large, Uniform Spheroids and Organoids,” Methods Mol Biol, vol. 2273, pp. 17–62, 2021, doi: 10.1007/978-1-0716-1246-0_2.

[2] T. Smit et al., “Characterization of an Alginate Encapsulated LS180 Spheroid Model for Anti-colorectal Cancer Compound Screening,” ACS Med Chem Lett, vol. 11, no. 5, pp. 1014–1021, May 2020, doi: 10.1021/ACSMEDCHEMLETT.0C00076.

[3] Liu H, Zang C, Fenner MH, Possinger K, Elstner E. PPARgamma ligands and ATRA inhibit the invasion of human breast cancer cells in vitro. Breast Cancer Res Treat, 2003. 79(1):63-74.

[4] Chavez KJ, Garimella SV, Lipkowitz S. Triple Negative Breast Cancer Cell Lines: One Tool in the Search for Better Treatment of Triple Negative Breast Cancer. Breast Dis, 2010. 32(12):35–48.