# Application of Cell-Mate™ 3D matrix to modeling artificial breast cancer and "normal" ductal tissues.

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## Abstract

In collaboration with BRTI Lifesciences, LLC., the UWRF TCIC has been engaged in testing and evaluating a new synthetic 3D tissue engineering matrix material product called Cell-Mate. This new material is based on a combination of hyaluronic acid and chitosan which yields a final matrix gel that enmeshes cells at relatively high densities to generate artificial tissues. In this series of studies, breast ductal adenocarcinoma cells (MCF-7) and "normal" breast ductal cells (MCF10A) were employed to generate significant artificial tissues (ATs) based on the application of Cell-Mate. MCF-7 ATs were generated from 40, 20 and 10 million cells respectively and ATs from MCF10A were generated from 20 and 10 million cells. In all cases, successful ATs resulted with significant areas of tissue or tumor-like architecture and distinct evidence of cellular differentiation as well as tumor cell progression. MCF-7 ATs generated evidence of tumor progression and eventual metastasis-related spheroid, cluster and single cell release. In the case of 40 million cell seeding loads, spheroid production occurred within the first week of culture while at 20 and 10 million cell loads the timing of spheroid generation/release was significantly longer at 2-3 weeks. However, within the first week, 20 million and 10 million cell loads did show definitive rounded features on the surfaces of the developing ATs. Interestingly, some cluster generation was seen in the "normal" MCF10A cell line ATs as well, but at a much lower level. Also in the case of MCF10A, shed cells formed monolayers in the bottom of culture wells which displayed differentiation-associated cells and colonies after 2-3 weeks of ATs development. These cells contrasted distinctly from those seen in the original culture monolayers which strongly supports the interpretation that ATs microenvironments induce pathway specific changes in cellular behaviors. Continuing studies are evaluating the morphology and marker expression profiles of tissues within the generated ATs as well as examining and comparing Cell-Mate generated MCF-7 spheroids in contrast to media-induced or hanging drop culture generated spheroids. Based on studies to date, we propose the application of Cell-Mate as an effective approach to modeling breast cancer tumors in-vitro and expect that this will open the door to better understanding of the role of microenvironments in tumor progression generally.


## Project Concept and Overview.

During the past several years our laboratory has been engaged in the development and application of new 3D cell culture technologies to model and characterize various developmental and pathologic tissues. In these studies we have established small-scale artificial tissues (ATs) or artificial tumor tissues (ATTs) from primary avian fetal cell and tissue isolates, porcine cardiac stem cells, human embryonic stem cells, approximately 25 ATCC cell lines (both "normal " and neoplastic) and several primary patient tumors from prostate, lung, colon, brain and breast cancers. In all of these studies, ATs were established and cultured over extended periods of time as continuous "tissue-like" 3D structures. In most cases, tissue survival extended to at least 9 months of continuous culture and in a few cases the cultures have been maintained for more than 2 years. Among the samples of patient tumors were a set of breast cancers which established artificial tumors that displayed characteristics reflective of the natural "tissue of origin" structure and morphology.

We next decided to focus our attention on modeling breast cancers in 3D using standard ATCC tumor cell lines, in particular, the ductal adenocarcinoma line designated as MCF-7. In a series of studies over several years, we have established methods and techniques in our laboratory to generate and test these complex 3D artificial tumors using various natural scaffold materials and also generated tumor spheroids by several methods. Together, these approaches have produced an effective toolset for us to explore both basic tumor development and biology as well as modeling metastatic-like behaviors which correlate very well with our early translational medicine observations of patient tumor samples.

In the normal human mammary gland, represented in Figure 1 (right), ductal cells generate a branching tube that terminates in blind sack-like features called acini and which themselves perform the secretory function of this organ. With 3D modeling techniques using natural de-cellularized extracellular matrix materials, MCF-7 adenocarcinoma cells can be made to generate most of these "normal" tissue features. In addition, such 3D structures can then give rise to spontaneous or induced spheroid structures that closely resemble metastatic tumor nodules.

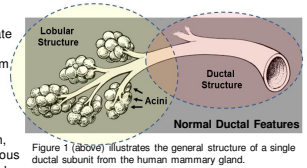



Figure 1 (above) illustrates the general structure of a single ductal subunit from the human mammary gland.

Until recently, the majority of the work done in our laboratory modeling various types of cancer and normal tissue generation in 3D cultures has focused on the application of several different natural matrix materials from basic ECM of marine origin to intestinal sub-mucosa and de-calcified spongy bone. All of which worked to varying degrees with a wide range of human, mouse, porcine, ovine and other cell types to generate complex and long-lived artificial tissues (ATs) or artificial tumor tissues (ATTs). Here we report on a new direction for matrix materials in our work, by applying the newly developed "Cell-Mate" chitosan/hyaluronate (CT/HA) blended matrix produced by BRTI Life Sciences LLC., based in Duluth, MN. This material represents a significant change of strategy for our program and addresses some potential short comings of the natural matrix products we have been using previously.

## Standard Cell-Mate 3D Matrix System Workflow

Unlike the natural matrix materials that have been our laboratory's previous standard 3D substrate, Cell-Mate is a defined material in which all the concentrations and specific components are carefully controlled with a high degree of precision. Although in this case, the components are still natural products as well, with CT being the major component of crustacean exoskeletons while HA is a major component of early developmental ECM in the normal body. HA is also expressed heavily during wound repair. Both these components are polysaccharides and afford cells numerous attachment points through receptors like CD44. This has some very intriguing implications, since CD44 is a common stem cell marker and directly ties into differentiation and cell cycle regulation pathways. Therefore, the response of tumor cells to this new matrix is of particular interest to our lab.

Figure 2 (left) illustrates the component of the basic Cell-Mate scaffolding kit. These include the powdered matrix within a 50ml conical centrifuge tube used for mixing. A standardized "rehydration" fluid and a custom concentration tube used to form the developing "cocoon" of matrix and cells. In our hands, we have found that a 250ul kit requires between 10-20 million MCF-7 or MCF10A cells to generate an effective tissue. Fewer than this number of cells generates a very loose matrix which fails to hold together over time in culture. This result is not surprising when it is understood that the cells actually become enmeshed into the matrix and form part of the overall early structure. Hence the stability of the developing tissue is directly effected by the cells present.

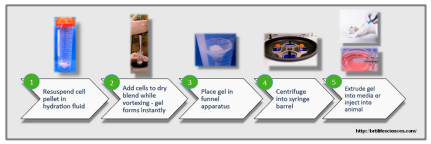



Figure 3 (right) displays the basic workflow used with the Cell-Mate kit to generate embedded cell/matrix scaffolds. The resulting scaffolds require a few days to fully form, during which some pockets of trapped air are commonly observed. By 5 days these are completely dissipated and the tissue begins to develop in earnest.

## Cell-Mate Based Tissue Modeling: MCF-7 Adenocarcinoma Cells.

In a recent series of experiments we set out to test MCF-7 breast adenocarcinoma cells in 5, 10 and 20 million cell density loads with Cell-Mate. As in our initial experiments, these resulting Cell-Mate based breast cancer ATTs began generating spheres and large rounded surface features suggesting regional distributions of spheroid generating tumor cells. However, interestingly, in these new experiments far fewer released spheroids were observed than in previous experiments (figure 7a-b, below), while the ATs still displayed large numbers on the overall structure surfaces. Additionally, in at least one case with 20 million cells loaded, a single extremely large rounded but non-releasing structure could be seen growing from the mass of tumor tissue (figure 4, below).

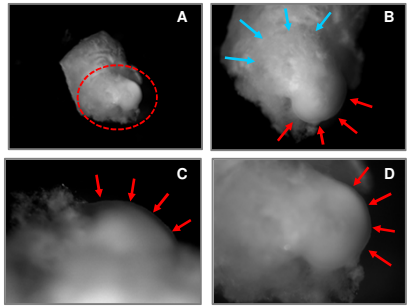



Figure 4a-d, (left) illustrates the outcome of 4 weeks growth of MCF-7 with 20 million cells loaded into a 250ul Cell-Mate scaffold. Here a single large mass of rounded tissue can be seen extending from a deeper region of tissue encompassing nearly a third of the overall matrix. In panel "A", the overall ATTs can be seen with a large very rounded mass extending out toward the bottom of the image (red circle). As this mass of tissue extends back into the scaffold, it is clear that there are various regions of cellular structure. This is seen more clearly in panel "B" at higher magnification (blue arrows). The surface of the mass is indicated by red arrows, as it is in panels "C" and "D". Each of these is the same rounded feature, but shown here 3 days apart as it developed. This rate of bulk expansion strongly suggests that the cells here are growing very rapidly. This point is particularly apparent in the size differential from panels "C" to "D".

## Cell-Mate MCF-7 ATTs Spontaneously Generate Spheroids.

During the course of several studies, we have determined that MCF-7 Cell-Mate ATTs not only form large tissue masses as shown above in figure 4a-d, but that these also generate regions where significant small (50-500um) spheres are formed and may be released into the media. In these experiments, there appeared to be a relationship between the loading cell number and tendency to generate larger numbers of these spheres.

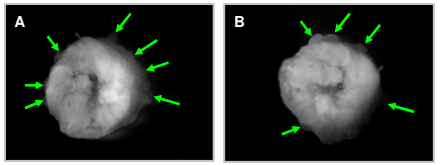



Figure 5a-b, (right) shows another MCF-7 Cell-Mate scaffold imaged from the end with two different angles of orientation. In this case, there is no single large mass seen, however, numerous smaller spherical structures are evident around the boundary of the tissue (green arrows). In addition, the varying shades of white tissue and grey areas strongly suggests regional differences in tissue development across the ATTs surface. It is possible that these represent areas of future spheroid formation. The central cavity seen in these images is a result of matrix placement in the spin tubes (see figures 2 and 3) during sample preparation.

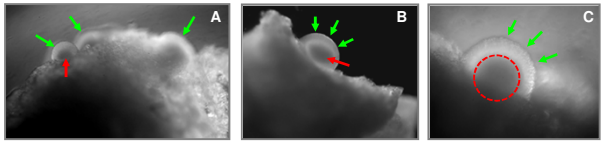



Figure 6a-c, (above) shows higher magnification images of spheroids forming in different regions on three MCF-7 Cell-Mate ATTs. In each of these cases, the spheroids are seen being generated from specific regions on each scaffold but not from other areas. In panel A, several spheres (green arrows) are being generated together while the intervening surface tissue is relatively flat and unremarkable at this stage. The sphere to the far left in this image is well focused and illustrates a common feature that we have observed in many MCF-7 derived spheres from various sources, including other 3D scaffolds, hanging drop cultures and those spontaneously generated from densely grown monolayers. This feature is a well defined central "core" (red arrow) and a thin-to-thick cortical layer. Generally, it is possible to focus through the sides of the cortex to image cells within this layer as can be seen here in panels 6b and particularly 6c. These cells commonly have a more organized appearance than the surrounding tissue surfaces as well, which clearly contributes to the distinctly smooth curvature observed.

In earlier studies using 40 million cells per 250ul cocoon, a clear pattern was observed of higher spheroid formation and release, leading to a wide range of sizes and complexities of these structures. We interpret this complexity to be an indicator of relative spheroid maturation. These data suggest that the formation of spheres by MCF-7 ATTs is highly tissue density dependent. This view is also supported by studies on natural scaffolds and in hanging drop cultures.

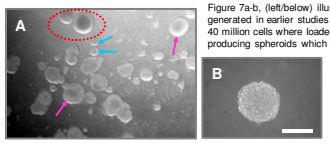
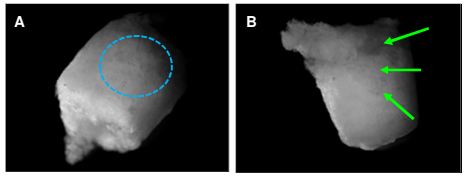



Figure 7a-b, (left/below) illustrates the yield of MCF-7 spheroid structures observed being generated in earlier studies using higher cell numbers to load the scaffolds. In this case 40 million cells where loaded into a 250ul kit and the resulting ATTs was very prolific in producing spheroids which were released into the media. Panel "A" shows the range of sizes as well as differences in the internal complexity of these structures. Spheres indicated by the blue arrows are approximately 100um in diameter, while those indicated by the purple arrows are approximately 400-500um. The smaller spheres shown also lack apparent internal complexity, while the largest ones (red circle) clearly have a core structure similar to that seen above in figure 6b and c. Panel "B" shows a close-up image of a smaller sphere (bar=100um).

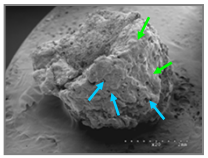
## Cell-Mate Based Tissue Modeling: "Control" MCF10A Ductal Cells.

In order to confirm that structural features and spheroids generated by MCF-7-based Cell-Mate ATTs were, in-fact, cancer-related in this new series of studies, the breast ductal epithelial cell line MCF10A was added to our experiments to generate control "normal" ATs. These were prepared with 10 or 20 million cells each, in either 500ul or 250ul preparations. Since this was a new cell line with Cell-Mate, the exact loading densities had to be derived by experimentation. The overall results remained basically the same in both cases with simply longer developmental times resulting from the lower cell densities. In all cases, cells and tissue-like structures could be observed in the ATs produced. Also, all ATs yielded significant shed cell monolayers and many of these also displayed evidence of cellular differentiation.

Figure 8a-b, (right) presents the results of MCF10A breast ductal cells grown in Cell-Mate based ATs for nearly one month of culture. In both cases, left-over fragments of matrix can be seen "extruded" from the ends of the tissue structure. Although there are clearly some cells associated with these features, these do not appear to be the result of tissue outgrowth, but rather, remaining cells from the embedment process. On the other hand, ATs surfaces can be seen in both cases to have a patterned distribution of darker and lighter areas within the blue circle and indicated by the green arrows respectively.




## MCF10A control ductal cells display evidence of complex tissue formation and even of potential secretory function.



MCF10A–based ATs began to show evidence of secretory products (figures 12a-b) following approximately 3-4 weeks of culture. This continued throughout the following culture periods, which have now extended to more then 7 months. At 7 months, a representative set of samples were harvested and examined by SEM. The resultant data clearly demonstrate that, in fact, these Cell-Mate based ATs display complex epithelial tissues and distinct tubular features which can only be interpreted as being actual ducts. No such clear-cut ductal structural details have been previously observed by our lab. In addition, many of these features contain materials which appear to be in the process of being secreted as a flocculent.

Figure 9, (above) shows a 7 month old MCF10A /Cell-Mate construct. The smooth epithelial nature of the surface can clearly be seen across most of the structure (green arrows). At the same time, an extensive series of complex openings in those surfaces is also evident across much of the structure (blue arrows).

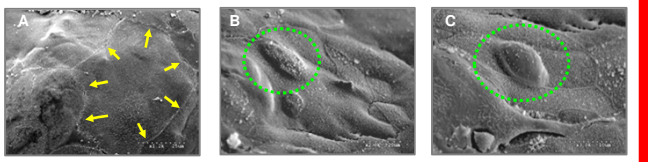



Figure 10a-c, (above) presents higher magnification views of the surface details on 7 month old MCF10A /Cell-Mate constructs. These images show smooth surface features, particularly in panel "A", which illustrates clear cellular junctions running along between the cells (yellow arrows). Panel "B" and "C" illustrate the presence of occasional bulging cells which appear periodically across the surface. These same cells have been seen in MCF10A 3D cultures using other scaffolding materials as well. In those studies, such cells appear to also have a direct relationship with the formation of ductal-like structures. Therefore, we hypothesize that this is the case here as well.

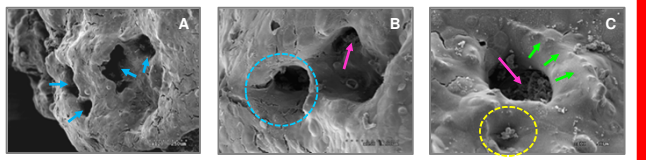
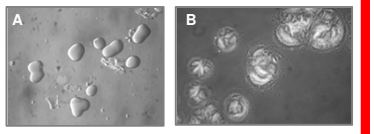



Figure 11a-c, (above) illustrates structural details of the abundant ducts seen emerging from 7 month old MCF10A /Cell-Mate constructs. In panel "A", we can see evidence of the complex nature of these ductal structures, with approximately 5-6 tubular features all gathered and exiting the AT in one general area (blue arrows). In panel "B" another cluster of ducts is viewed in such a way as to visualize content within at least one (purple arrow) and suggestions of ductal branching in another (blue circle). Panel "C" also illustrates the presence of a flocculent content (purple arrow) as well as details of the "lip" region of the duct (green arrows). Also shown here is an new duct apparently forming as the surface regresses/collapses into the structure (yellow circle).

Figure 12, (right) presents two forms of evidence which suggest that MCF10A Cell-Mate ATs are actively secreting fatty materials into the culture media. In panel "A" globules of materials are seen in the bottom of the well. These are characteristically smooth and highly retractile with little or no internal detail being seen. Panel "B" on the other hand shows lipid droplets that are floating on the surface of the media, forming a thin layer across wide areas of the well. Both of these features appear to have a high lipid content and are not cellular in nature.




## Summary and Future Directions.

In this poster we have demonstrated that the new Cell-Mate 3D Matrix material can and does work as a viable scaffolding for the growth of ductal adenocarcinoma as well as "normal" breast ductal epithelial tissues. In both cases, the resultant structures contained significant tissue features consistent with the cancerous or normal context of the original cells. In the case of MCF-7 adenocarcinoma cells, we observed the generation of large rounded masses of presumed tumor tissues at lower cell seeding densities of 5-20 million cells and abundant generation and shedding of presumed macro-metastatic spheroid structures ranging from ~50-500um in diameter from ATs seeded with 40 million cells. For MCF10A "normal" ductal cells, we saw the generation of excellent epithelial tissues across the ATs as it matured over time. This was followed by the formation of large numbers of tubular ducts with evidence of secretion from these features, potentially including the generation of milk fats. Ongoing and future studies will examine and further define/characterize each of these observed features as well as seek to understand the in-vitro developmental pathways involved.

## Background Literature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### Acknowledgements

This research was supported in part by UWRF Foundation Grant support to the Cellular Imaging and Analysis Center, College of Arts and Sciences / College of Agriculture, Food and Environmental Sciences Collaborative Laboratory Modification grants, the Department of Biology at the University of Wisconsin-River Falls and the TCIC Annual Budget. Imaging support provided by the UMinn Imaging Center, St Paul, MN.