SurFACTS in Biomaterials

Spring 2013 | Volume 18 Issue 2

Hydrophobic Inserts for the Production of Tumor Cell Aggregates and Embryoid Bodies

By Briana N. Vogen, Laurie R. Lawin, Leandro Forciniti, Daniel E. Guire, and Tahmina Naqvi; Innovative Surface Technologies, Inc. (ISurTec®),

The cost of drug development greatly increases as a drug or therapy moves from the initial stages of biochemical assays to cell-based assays, and especially to animal models and clinical trials.[1] The overall success rate for oncology products in clinical development is only ~10%, and the approximate cost of bringing a new drug to market is over $1 billion US dollars.[2] A more accurate predictive system of cell culture is needed to decrease the cost of drug development while simultaneously increasing the overall success rate at the initial stages of drug development and clinical testing. In recent years, in order to alleviate unnecessary animal suffering and decrease research costs, considerable effort has gone into growing eukaryotic cell aggregate(s) to simulate such cell growth in vivo.

Classically, in vitro eukaryotic cells have been cultured as a monolayer on two-dimensional (2D) surfaces made of synthetic materials, such as glass and plastic. However, it is generally accepted that in vitro 3D cell aggregate(s) are physiologically more relevant in comparison to 2D cell monolayer(s), and thus can provide a more accurate precursor model in simulating in vivo animal studies. In the last decade, various studies have demonstrated that in vitro 2D cell monolayers do not accurately represent the in vivo microenvironment, nutrient intake, and the biochemical processes involved in protein and gene expression. Many different in vitro methods of growing 3D cell aggregate(s) have been evaluated to accurately simulate in vivo models used in research studies.[3-6] However, producing an easy-to-use product that facilitates the formation of 3D cell aggregate(s)

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economically has proven to be a difficult design challenge.

In biological and medical research, examining the formation of three-dimensional (3D) aggregate(s) of eukaryotic cells, such as tumor cell aggregate(s) and embryoid bodies (EBs), is important for the evaluation of various drug screenings, biological compatibility, and cell therapies as well as determining gene/protein expression profiles and engineering cell tissue for various purposes. Single, uniform-sized 3D cell aggregates are desirable for repeatability in experiments. The generation of a single spheroid per well allows for the screening and testing of biochemical compounds, biological agents, and infectious organisms and also allows for toxicity evaluations. In the case of embryoid bodies (EBs), spheroid size is linked to cell differentiation potential.[7] In widely used hydrophilic dishes for spheroid culture, agglomeration of spheroids is an area of concern. Large agglomerates can produce their own microenvironment, within which cells produce their own growth factors. This makes it harder to control the culture environment and determine the effects of exogenous factors. Therefore, the use of inserts where a single EB is present per well offers an effective solution to prevent agglomeration of EBs. In addition, the formation of EBs in a uniform and reproducible manner with regulated homogeneity in morphology and differentiation status is desirable for research purposes. Indeed, for screening purposes, uniform-sized EBs have been shown to have synchronous differentiation potential.[4,7]

An in vitro technique widely used to grow eukaryotic cell 3D aggregate(s) is referred to as the Hanging Drop Method. This method has proven useful both for growing cancer cell lines into tumor spheroids and for growing embryonic stem cells into EBs. To practice this method, a cell culture medium having a known concentration of cells is added as drops to the underside of a glass Petri dish cover. Sterile water is then placed in the mating bottom portion to maintain humidity within the dish. The dish cover with the suspended drops containing cells in culture medium is gently inverted and is placed atop the mating bottom portion. Due to gravity acting on the cell culture drops suspended from the inverted surface, the cells, being heavier than the medium, settle from the cell suspension in the medium to the bottom of the meniscus of each hanging drop. This unique configuration allows for only the top part of the medium (e.g., the part hanging from the Petri dish lid), which is cell-poor, to be in contact with substrate, and, as a result, the cells form 3D aggregate(s) in the cell medium and do not come in contact with any synthetic surface (e.g., glass or plastic) used in 2D monolayer techniques. This method produces a single aggregate per drop with an efficiency of near 100%.[6]

However, there are multiple drawbacks to the Hanging Drop Method. For example, the drops of medium are held on the surface of the Petri dish only by surface tension and adhesion forces, resulting in drop size being limited to a volume of 50 µL or less to resist the gravitational force pulling down on each drop. Another drawback to this method is that the drops are accessible only if the Petri dish lid containing the drops is gently inverted. Thus, it becomes difficult to change the cell culture medium and to periodically observe the growth of the cell aggregate(s) using a microscope. Furthermore, the

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agitation caused by inverting the dish can easily cause the drops to run together or fall into the bottom half of the Petri dish. Additionally, if the plate is bumped or rocked even slightly, the hanging drops can easily fall into the media below them, thus making the recovery of the cells/aggregate(s) contained in the drops highly problematic. On average, only 50-60% of aggregate(s) can be recovered using this method. Moreover, this method is inherently incapable of large-scale production[8].

Our product, ISurSphere™ inserts, is designed to circumvent these drawbacks. ISurSphere™ inserts consists of an array of conical inserts for a 96-well plate, each with a viewing hole located at its apex, shown in Figure 1. The inner surface of the ISurSphere™ inserts is hydrophobic; thus, when any aqueous medium is applied above the viewing hole of the insert, a near-spherical culture drop is formed. Cell aggregate formation and growth can be observed through the viewing hole at any time using a microscope.

Advantages

1. The cell culture-containing drops are not easily dislodged by vibration or movement of the cell culture plate. Even if they are dislodged due to violent vibration or jostling, the cell aggregate(s) could easily be recovered from the well below.
2. Fusion of cell culture drops during growth or movement of the 3D cell culture insert is not possible.

Figure 1. Schematic of a tray of conjoined ISurSphere™ inserts fitting into a 96-well plate for 3D cell culture.

3. Unlike the Hanging Drop Method and its adaptations, the growth medium does not need to contain serum.
4. Direct microscopy can be conveniently used to observe and monitor the growth of 3D cell aggregates.
5. The ability to generate a single spheroid per well while providing superior size control over the spheroid.
6. Replenishment of the medium bathing the cell aggregates can be performed easily.
7. Medium replacement can also be streamlined by employing automated dispensing devices for high throughput applications.
8. The working drop size can be increased well beyond 50 µL, hence minimizing evaporation and medium change issues.

Potential Applications

A wide variety of cell types and cell lines can be employed for the formation, growth, maturation, and observation of cell aggregate(s) using this product. For example, 3D stem cell aggregate(s) for adult, embryonic, and induced pluripotent stem cells can be formed for directed differentiation and screening studies. 3D cell aggregate(s) from liver and kidney cell lines can be tested for toxicity screening. Several tumor cell line aggregate(s) such as cervical cancer (HeLa), colon cancer (RKO, HT29), prostate cancer (ALVA-31 and PPC-1, PC-3), breast cancer (MCF7, T47D), and ovarian cancer (A2780) can be tested for the effect of small molecule and drug screens. In addition, this product can also be used to support the formation of spheroids from widely used and well-characterized cell lines such as CHO (Chinese Hamster Ovary cells), HEK (Human Embryonic Kidney cells), HUVECs (HUman Vascular Endothelial Cells), and BAEC (Bovine Aortic Endothelial Cells).

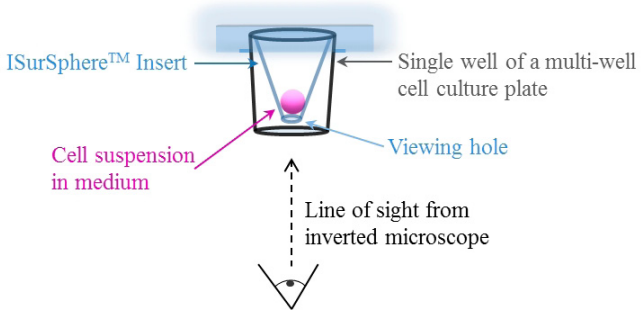

Figure 2. Schematic diagram of a single ISurSphere™ insert placed in a single well of a multi-well cell culture plate depicting a drop of cell suspension in growth medium above the viewing hole.

Experimental Results

Thus far, we have been successful in producing aggregates with nearly 100% formation efficiency. Throughout formation and growth of the single aggregates in ISurSphere™ inserts, the aggregates remain near the middle of the insert. This is particularly helpful for both recovery of the aggregate(s) and microscopic observation through the bottom of the viewing hole.

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Figure 3 shows HeLa cell aggregate formation and growth throughout a 10-day growth period. The aggregate remains within the center of the insert throughout this growth period (Figure 4). As for the longevity of 3D cell culture, both BAEC and HeLa 3D aggregates have been cultured for over 20 days.

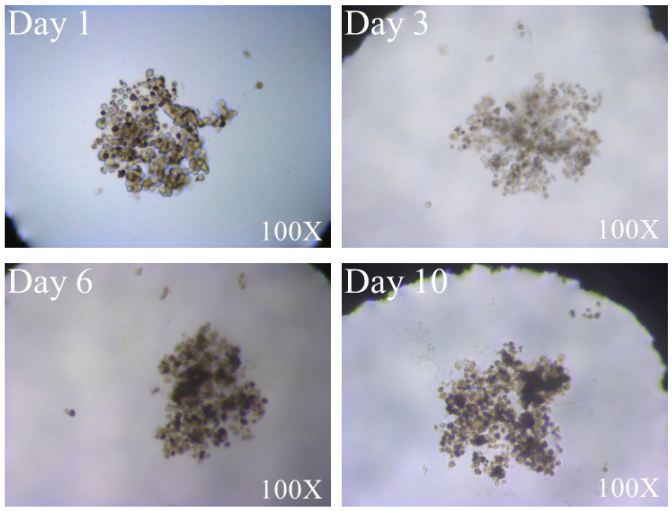

Figure 3. Optical micrographs of HeLa cells (~1100 cells per device) in ISurSphere™ cell culture inserts over a 10-day 3D growth period.

Acknowledgements

The authors would like to gratefully acknowledge funding from the National Science Foundation (NSF) and the American Society for Engineering Education (ASEE) in helping to support the development of this product (NSF Grant #0848918).

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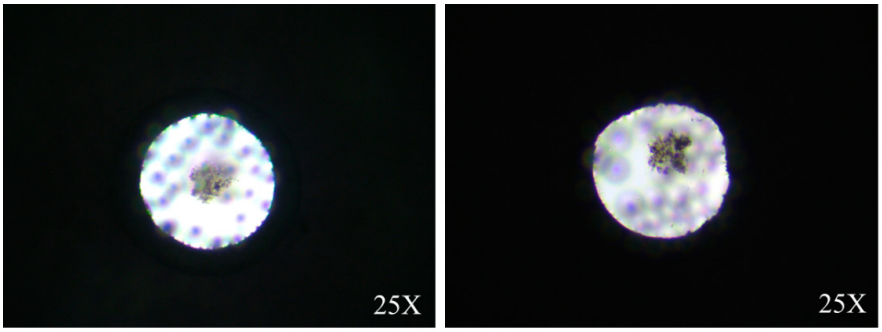

Figure 4. HeLa cells (~1100 cells per device) in two different conical inserts after a 10-day growth period.

Cationic α-Helical Polypeptide Template for Nonviral Gene Delivery

*By Rujing Zhang, Hua Lu, Nathan P. Gabrielson, Lichen Yin and Jianjun Cheng**
Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign

Introduction

Gene therapy facilitates the treatment of human disease through the transfer of genetic material (e.g. DNA) into cells.[1] Due to DNA's inability to pass through cell membranes, the development of effective delivery vectors is crucial. As a safer alternative to viral vectors, nonviral vectors have attracted more and more interest. Polypeptides, such as cationic poly-L-lysine (PLL), were the first set of materials considered for use as nonviral gene delivery vectors.[2] However, despite its ability to bind and condense anionic plasmid DNA, PLL and its modified variants have suffered from generally low transfection efficiency.[3-5] As such, the function of peptide-based materials has gradually shifted to other roles relevant to transfection. For instance, the cell penetrating peptides (CPPs) penetratin,[6] transportan,[7] and TAT[8] have found use as component materials that, when incorporated into delivery vectors, lead to increased internalization, improved endocytic escape and overall better transfection efficiency.[9] While effective in promoting membrane destabilization as part of a larger vector, CPPs are often too small or lack an adequate cationic charge density to function as stand-alone gene delivery vectors. Ideally, the structural characteristics of CPPs which facilitate their membrane destabilization (i.e. helical secondary structure) would be incorporated into the design of polypeptide vectors to improve their transfection performance.[10] Unfortunately, all cationic polypeptides (PLL, modified PLL, or other polypeptide electrolytes) adopt random coil structures due to intra-molecular side chain charge repulsion.[11,12] In this article, we summarize our efforts in constructing cationic α-helical polypeptide vectors for nonviral gene delivery.

Ionic polypeptides with unusual helical stability

Helical structure plays an important role in the interaction between CPPs and cell membranes. Similarly, cationic charge plays an important role in facilitating the interaction between nonviral vectors and DNA. However, incorporating charged groups in polypeptide chains typically leads to reduced helical stability because of increased side-chain charge repulsion, reduced side-chain hydrophobicity and the disruption of intra-molecular hydrogen bonding.[13] We recently developed a strategy, based on controlled polymerization of α-amino acid N-carboxyanhydrides (NCAs) that was previously reported by us,[14] to successfully prepare water-soluble, ultra-stable α-helical polypeptides by elongating the charge-containing side chains to position the charges distally from the polypeptide backbone (Figure 1A).[15] We reasoned that as the charge was moved farther away from the backbone, its disruptive effect on the helicity of the molecule would be lessened. Moreover, as the side chains were lengthened, the increased

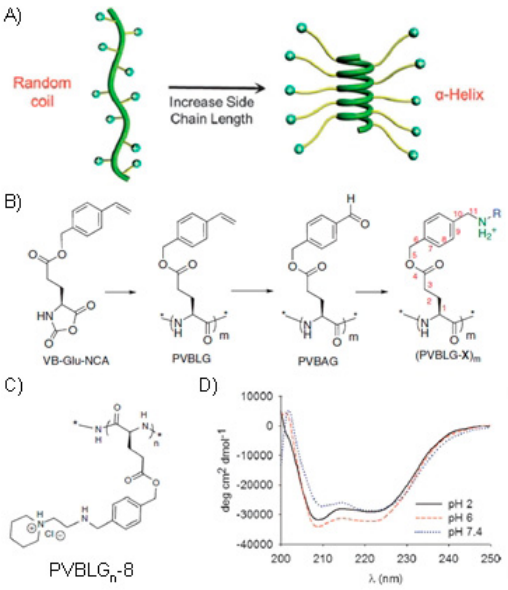

Figure 1. A) Polypeptides with charged side chains and the random coil to helix transformation in response to elongated side chains. B) Reaction scheme for the synthesis of PVBLG-X polypeptides. C) Chemical structure of PVBLG$_n$-8. D) CD analysis of PVBLG$_{267}$-8 at pH value of 2, 6 and 7.4.

hydrophobic interaction between adjacent side chains would serve to strengthen the overall helical structure. As a net result, we believed such polypeptides would simultaneously maintain water solubility as well as stable helical structures. Our subsequent studies concluded that when the charged group was placed 11 δ-bonds (Figure 2B) away from the polypeptide backbone, the corresponding polypeptide showed stable helical structures with high helical content (>90%) within a broad range of pH (from 1 to 10). In addition, the helical structure was also demonstrated to be resistant to various environment changes including increased temperature, ionic strength and typical protein denaturing agents.

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Reactive and bioactive cationic α-helical polypeptide template for nonviral gene delivery

We have combined our experience with controlled NCA polymerization and helical polypeptides to generate nonviral gene delivery vectors that are sufficiently large and positively charged to bind and condense DNA, yet also possess the helical structure seen in many CPPs[16]. As part of our initial work, a library of cationic α-helical polypeptides was synthesized and screened in attempts to identify particular amine side chains that yielded helical molecules with the appropriate balance of hydrophilicity (i.e., DNA binding strength) and hydrophobicity (i.e., endosomolysis) to mediate efficient gene delivery. The top-performing material, named PVBLG-8 (Figure 1C), was identified and confirmed in several cell lines (12-fold improvement over a commonly used transfection reagent, polyethylenimine). Circular dichroism analysis (CD) confirmed the stable helical conformation of PVBLG-8 (Figure 1D). A random coil analogue, PVB-DL-G-8, was shown to have negligible transfection efficiency, thereby highlighting the importance of PVBLG-8 helical structure (Figure 2). Further characterization studies suggested that the success of PVBLG-8 was largely due to its ability to disrupt endosomes. In view of this reactive polypeptide template, our current studies are focused on developing novel molecular architectures based on PVBLG-8 (e.g. block co-polymers, star polymer, etc.)[17] as well as exploring the potential for the material to mediate siRNA delivery[18] and protein delivery and in vivo gene delivery.

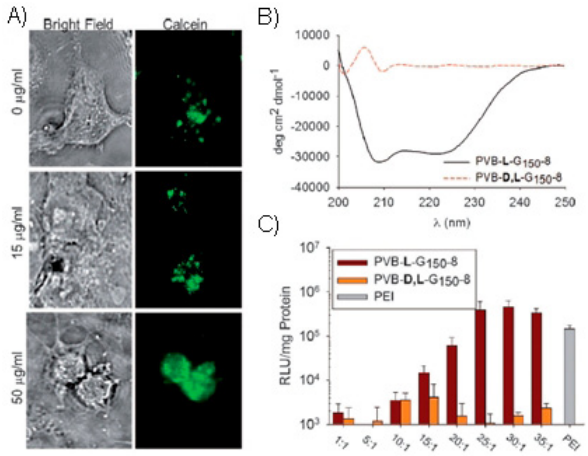

Figure 2. A) Calcein uptake in COS-7 cells treated with various concentrations of $PVBLG_{267}$-8. Calcein is unable to cross intact membranes. As the amount of $PVBLG_{267}$-8 in the extracellular medium is increased, the intracellular fluorescent signal becomes more diffuse, thus indicating membrane permeation and non-endocytic calcein uptake. B) CD spectra of helical PVB-L-G_{150}-8 and random coil PVB-D,L-G_{150}-8 in water. C) In vitro transfection of COS-7 cells with PVB-L-G_{150}-8 and PVB-D,L-G_{150}-8 polypeptides. 25 kDa PEI was included as a control.

Acknowledgments

We acknowledge financial support from the NSF (CHE-0809420) and the NIH (NIH Director's New Innovator Award 1DP2OD007246, 1R21EB013379).

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Regulatory Meanderings

By Phil Triolo, PhD, RAC, SurFACTS Regulatory Editor

On our way down to Mexico for a family vacation over my daughter's spring break, we changed planes in Phoenix. As my intent had been to finish off this article in transit, my mind was on *SurFACTS* and the Surfaces Foundation. As we touched down in Phoenix I recalled with great fondness the Scottsdale Meetings. Yes, their timing was miserable; I didn't like giving up the 3-day Labor Day weekend to attend a technical meeting. But, I learned to tolerate the inconvenient date as the atmosphere was superb for conducting business. I remember excellent pool sessions and chats that extended past sundown conducted in the cool evening breeze. When are we going back?

I spent a good bit of my vacation, unfortunately, ironing out some problems with a technical file for a start-up company. For those of you who are about to, or are in the process of placing a medical device or combination product on the market in the US or EU or Timbuktu, here are a couple of practical suggestions that will make it easier for you, or contract help, to organize your information into a submission that has a good chance of being approved, CE Marked, licensed, etc.

DO:

- Title and number your documents in an orderly and comprehensible fashion so that they can be easily identified and located;
- Archive or in some way distinguish old versions of documents from new, current versions, and only keep the current versions in your working file of technical documentation (that you provide to third parties for evaluation or incorporation into a technical file, e.g.);
- Keep an electronic version of all files in a backup file stored somewhere safe, so when your computer gets stolen on your trip to Mexico (no, mine didn't) you will still have the information. A flash drive can work just fine for this purpose;
- Organize those third-party documents in a file with either your or their document identifier (e.g. lab number) appearing in the title;
- Create specifications early in the development process;
- Name the device and use the name consistently, especially in labeling;
- Define the scope of the project; what products will you be selling? Don't forget those purchased accessories. They count, too;
- Early in the design phase, document the intended and indicated use of the product. Without these, it is impossible to determine if the device is safe and effective, or evaluate risks and benefits;
- Allow time for the implementation of a quality system that defines the regulatory activities that need to be performed, as well as technical tasks;
- Provide answers to questions in a reasonable timeframe. Otherwise, expect to pay twice as much as anticipated for contracted services, as it takes time to get "up to speed" to incorporate new information if the time lag is too great. One week is enough time to provide reasonable answers to reasonable questions; and
- Provide diagrams and photos of your device and accessories to aid in an understanding of its operation.

DO NOT:

- Include irrelevant or outdated information in the packet supplied to regulatory contractors, and especially not regulatory authorities;
- Assume that because you are familiar with the device and its use, others will be, too - especially if no good technical descriptions are provided in writing and drawings are sketchy;
- Attempt to be cute with words to avoid having to meet regulatory requirements. I've been a reviewer. These attempts only aggravated me and made me want to find and point out those little annoying deficiencies;
- Attempt to extensively reword the text of technical writers, unless the wording is false, misleading, or misrepresents your device or its use;

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- Make claims just to see if they will be accepted, unless there is at least some data to support them;
- Assume that you can conduct accelerated aging studies at 75° C to shorten the time to complete studies to validate a 3-year shelf life claim to 6 weeks;
- Assume that because you use the "same" materials that are used in other medical devices, you can use them in yours without any additional testing; or
- Forget that the device has to be packaged and shipped, and you'll have to demonstrate that it still meets specifications after exposure to typical shipping and storage conditions.

Record keeping can be sloppy in a small work environment when there are few qualified internal reviewers. Try to keep information well organized and even pay for some outside reviews. The investment will pay off down the road in shortened submission preparation and review times.

Functional Language in Apparatus Claims

By Colin Fairman, SurFACTS Intellectual Property and Legal Editor

A claim term is functional when it recites a feature "by what it does rather than by what it is" (e.g., as evidenced by its specific structure or specific ingredients). There is nothing inherently wrong with defining some part of an invention in functional terms. In re Swinehart, 439 F.2d (CCPA, 1970) 210, 212. Manual of Patent Examining Procedure; 2173.05(g).

Functional Limitations

We all do it, at least once, putting functional language in an apparatus claim: At times, in an attempt to provide distinguishing characteristics, functional claim language is useful in carving around the prior art.

However, in some instances the use of functional claim language results in a rejection from the patent examiner on the basis of lack of written description and/or enablement § 112. (See this column "One Size Does Not Fit All" Summer 2012)

> *Claim 24 was at issue in Swinehart.*
>
> *24. A new composition of matter, transparent to infra-red rays and resistant to thermal shock, the same being a solidified melt of two components present in proportion approximately eutectic, one of said components being BaF2 and the other being CaF2.*

In this claim, at issue in *Swinehart*, I have underlined the functional language. The Examiner rejected the claim under § 112 on the grounds that the claim fails to properly point out the invention. This claim in reciting "transparent to infrared rays" is thus improperly functional. During administrative appeal, the Appeal Board concurred:

> *Claim 24 stands rejected as improperly functional in that it distinguishes over the unsatisfactory material of appellants' figure 3 merely in the functional term "transparent to infrared rays." We agree with the Examiner in this respect, as transparency of the claimed material cannot be treated as an inherent, characteristic property, in view of the fact that the composition of appellants' Example V (figure 3) lacks this property, yet is made of the same materials as appellants' Example I. * * * This claim is not the type covered by a proper functional limitation pursuant to 35 U.S.C. 112, since the language in question does not define a means or a step, or a distinguishing ingredient.*

In the case of *Swinehart*, the question asked by the examiner is how specifically is "transparent to infra-red rays" quantified? The functional language used in *Swinehart* is unique to the invention embodied in the eutectic crystal. However, other functional language is important in identifying the invention without being defining as part of the apparatus.

Take, for example, a drug that is given in an "effective amount" a very common phrase in drafting pharmaceutical and nutraceutical claims. However, as recently as 1970, Examiners were rejecting claims with this phrase because dosage amounts were not provided in the claims. In re Halleck, 422 F.2d 911, 164 USPQ 647 (CCPA 1970). Whether "an effective amount" is indefinite or not "[T]he proper test is whether or not one skilled in the art could determine specific values for the amount based on the disclosure." *Id.*

When the court construed the limitation "transparent to infra-red rays" it construed them in light of the specification which provided three charts showing the percentage of infrared transmission through a "window" made from the eutectic composition." *Id.* The charts showed a maximum transmittance of about 80% IR light but also showed the amount of transmittance was, to some extent, dependent on the particular methods used to make the eutectic crystal.

However, in allowing the claims the court provides:

> *The record before us establishes that prior art compositions are substantially opaque to infrared rays. Appellants have produced a composition which is substantially transparent to such rays. Such a composition is conceded to be novel. It is true that the figures reproduced in the specification indicate that the degree of transparency varies depending on such factors as the conditions employed in producing the crystal, the thickness of the crystal and the particular wave length of the radiation transmitted. However, in all cases a substantial amount of infrared radiation is transmitted. We do not read appellants' disclosure as suggesting that only certain degrees of transparency to infrared are comprehended within the teaching there given. It follows that when appellants' claim is read in light of that disclosure the limits it purports to define are made sufficiently clear.*

Thus, in *Swinehart*, by using functional claim language claims were able to encompass crystals that were transparent in the IR spectrum as measure by about any composition that was opaque to IR wavelengths.

"Functional terminology may render a claim quite broad.

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By its own literal terms a claim employing such language covers any and all embodiments which perform the recited function." *Swinehart.*

So, why not just use functional language? First, functional language must still satisfy the requirements of 112, first paragraph with regard to written description and enablement. Second, the use of functional language may indeed result in claims that encompass art that would anticipate or make obvious.

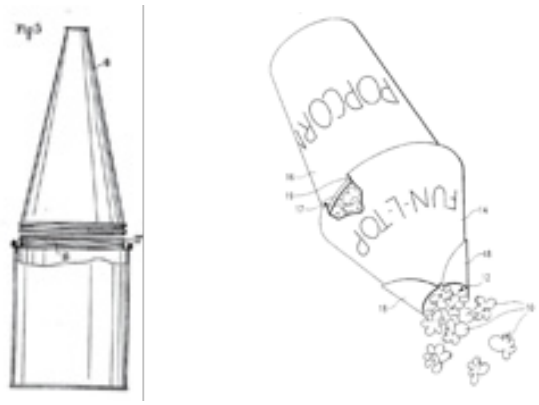
Consider, In Re Schreiber, 128 F.3d 1473 (Fed. Cir, (1997). Claim 1 provides:

> *A dispensing top for passing only several kernels of a popped popcorn at a time from an open-ended container filled with popped popcorn, having a generally conical shape and an opening at each end, the opening at the reduced end allows several kernels of popped popcorn to pass through at the same time, and means at the enlarged end of the top to embrace the open end of the container, the taper of the top being uniform and such as to by itself jam up the popped popcorn before the end of the cone and permit the dispensing of only a few kernels at a shake of a package when the top is mounted on the container.*

In rejecting claim 1, the Examiner found the same "conical top" could be identified in the prior art of Harz which was directed to a top for pouring oil.

While Schreiber denied that popcorn could be dispensed at only a few kernals at a time from the top of Harz, the court in any case found that Harz anticipated Schreiber. Claim 1 of issued patent 6,431,415 to Schreiber provides:

> *1. A dispensing top for passing only several kernels of a popped popcorn at a time from an open-ended container filled with popped popcorn, having a generally conical shape and an opening at each end, the opening at the reduced end being of a diameter greater than one inch and allows several kernels of popped popcorn to pass through at the same time, and means at the enlarged end of the top to embrace the open end of the container, the taper of the top being uniform and such as to by itself jam up the popped popcorn at the end of the cone and permit the dispensing of only a few kernels at a shake of the container when the top is mounted on the container.*



Harz *Schreiber*

Thus, after considerable expense taking the rejection to the Board of Patent Appeals and then to the Court of Appeals for the Federal Circuit, claims relying on functional language were maintained as rejected and objective limitations allowed patentability. This would have been a very expensive resolution.

In sum, what is the best practice? In my opinion, one of the most creative things I get to do is claim the same object different ways. That means, as long as I have support for various alternative forms of claiming, why not provide independent claims that have both functional and structural limitations? There may be ways to limit popcorn from exiting a funnel that do not rely on the actual size of the funnel opening which arguably would have been encompassed by the rejected claim. However, such alternative embodiments should be clearly and positively disclosed in the specification such that claims to such embodiments will not be rejected as lacking adequate written description.

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