Cytotechnology **36:** 33–39, 2001.

Advanced Granulation Technology (AGT™)

An alternate format for serum-free, chemically-defined and protein-free cell culture media

Richard Fike, Barbara Dadey, Richard Hassett, Robert Radominski, David Jayme* & David Cady

Cell Culture Research and Development, GIBCO/Invitrogen Corporation, Grand Island, NY 14072, U.S.A.
(Author for correspondence; E-mail: david.jayme@invitrogen.com, Fax: (+1) 716 774 6996)*

Received 4 January 2001; accepted 30 April 2001

Key words: biopharmaceutical production, complete formulation, granulated media, novel format, nutrient media, protein-free, serum-free

Abstract

To overcome limitations of conventional milling technology, we investigated the application of fluid bed granulation for the production of dry-form nutrient media. Serum-free, protein-free and chemically-defined specialty media were produced in granulated format and compared with identical formulations manufactured by conventional methods. HPLC analysis of multiple lots of granulated materials demonstrated that biochemical constituents were precisely and homogeneously distributed throughout the granules and that nutrient levels were comparable to conventional formats. Comparison of medium performance in cell proliferation and biological production assays demonstrated equivalence with reference media. The fluid bed granulation process meets pharmaceutical quality requirements and may be applied to a broad range of nutrient formulations required for bioproduction applications.

Abbreviations: AGT, advanced granulation technology; CD, chemically-defined; CHO, Chinese hamster ovary; CPE, cytopathic effect; DPM, dry powdered medium; EGF, epidermal growth factor; HEK, human embryonic kidney; HPLC, high performance liquid chromatography; PFU, plaque forming units; AcNPV, recombinant *Autographa californica* nuclear polyhedrosis virus; SFM, serum-free medium; TCID, tissue culture infective dose.

Introduction

Due to the risk of introducing foreign antigens or adventitious contaminants into biopharmaceutical processes through use of animal sera and undefined protein additives, serum-free, chemically-defined and protein-free nutrient media have become prevalent for cell culture bioproduction applications (Barnes, 1987; Murakami, 1989; Glassy et al., 1988). We recently developed specialty media formulations that support high density cell proliferation and biological productivity for key biotechnology applications. However, throughout the industry, it has been technically challenging to manufacture such specialty formulations in a powdered medium format with potency and performance equivalent to liquid formats. Critical limitations to conventional milling processes include: (a) potential cross-contamination and health concerns associated with dust generation; (b) lengthy and incomplete processes of powder solubilization and dissolution; (c) need to isolate 'sensitive' constituents from the basal formulation and to add them separately to the hydrated powder; (d) difficulty in achieving homogeneous distribution of minute formulation components; and (e) need for post-formulation pH and osmolality adjustment (Jayme et al., 2001).

As an alternative option to incorporate more desirable characteristics to cell culture media, we investigated the process of fluid bed granulation that is familiar to the pharmaceutical industry, but novel to nutrient medium manufacture. This AGT™ (Advanced Granulation Technology) nutrient media format (compared with the conventional ball-milled dry powdered medium format): (1) is not dusty when added to formulation tanks; (2) hydrates and dissolves readily; (3) is a complete formulation, i.e., does not require

independent growth factor, trace metal or lipid supplements; and (4) already includes sodium bicarbonate and requires no pH or osmolality adjustment upon rehydration.

We previously reported the ability to convert CD-CHO, (a chemically-defined, protein-free medium designed for CHO bioproduction applications (Gorfien et al., 1997) to the granular AGT^{TM} format (Jayme et al., 2001). This preliminary publication confirmed the concept of AGT^{TM} and demonstrated biochemical and biological equivalence with conventional formats. We have also shown the ability to scale up manufacture of CD-CHO Medium in the AGT^{TM} format from 2 kg quantities up to 500 kg production scale quantities with supporting constituent homogeneity and stability, cell growth, and biological production data (Radominski et al., 2001).

In this article, we expand the scope of AGT^{TM} format to additional specialty serum-free and protein-free formulations and their corresponding biotechnology applications: (a) VP-SFM for Vero cell growth and virus production (Price and Evege, 1997); (b) CD-Hybridoma Medium for hybridoma growth and monoclonal antibody production (Gorfien et al., 2000); (c) 293-II Medium for HEK 293 cell growth and adenovirus production (Epstein et al., 1999); and (d) SF-900 II Medium for insect cell growth and protein production using the baculovirus expression vector system (Godwin and Whitford, 1993). We compare the biochemical and biological results of these granulated media with reference media prepared using conventional manufacturing processes.

Materials and methods

AGT^{TM} process

The basic process used to produce AGT^{TM} formulations is fluid bed granulation, an operation used by the pharmaceutical industry for about 30 years to produce granulations used in tableting and to produce the coated beads used in time release capsules for oral dosage forms (Kadam, 1991). Figure 1 illustrates the schematic flow of air and materials within a fluid bed granulation chamber. Fluid bed granulation involves suspending a powder mixture in an upwardly moving column of heated, conditioned air flowing through a vertical chamber. Water and various aqueous solutions are sprayed onto the suspended powder within the chamber, causing individual particles to adhere and form aggregates that are quickly dried in the dehumidified air column (Parikh, 1997). These aggregates form a porous lattice-type structure that is readily wetted when added to water during media reconstitution. Trace ingredients are sprayed onto the powder as aqueous solutions, allowing more homogeneous distributed throughout the powder mass than if milled by conventional means. The AGT^{TM} test formulations were produced using a Niro MP1 fluid bed granulation apparatus located at our Grand Island, NY facility. All medium constituents were combined in the AGT^{TM} granules, including sodium bicarbonate and other buffers, and the formulation was configured to achieve a targeted pH value post-reconstitution. Granulated media were each added to water, stirred for 15 min, and filtered without any further additions or adjustments to pH or osmolality.

Nutrient medium performance assays

Granulated media were tested in cell growth and biological production bioassays. The following cell lines were used to evaluate the indicated media: Vero cells in VP-SFM, AE-1 murine hybridoma cells in CD-Hybridoma, HEK 293 cells in 293-II SFM and Sf-9 cells in SF-900II. Cells were cultured in tissue culture flasks using standard incubation protocols for each cell type. Cell counts were determined using a Coulter Counter and viability was assessed by vital dye exclusion.

VP-SFM performance was determined by evaluating Bovine Reovirus Type 3 production using TCID50 for virus quantification (Mahy and Kangro, 1996) with cytopathic effect (CPE) as evidence of infectivity. CD-Hybridoma Medium performance was monitored by quantifying relative monoclonal antibody production. Antibody was adsorbed onto an HPLC-based protein G affinity column (Perseptive Biosystems) and quantitated spectrophotometrically at 220 nm. Biological performance of 293 SFM was monitored by assaying for Human Adenovirus type 5 production, using TCID50 quantification of cytopathic effect as evidence of infectivity.

SF-900II performance was assayed by plaque production. Serial dilutions of stock virus (rAcNPV) were added to adherent Sf-9 cells and plaques were counted following a 5 day incubation in agarose overlay culture.

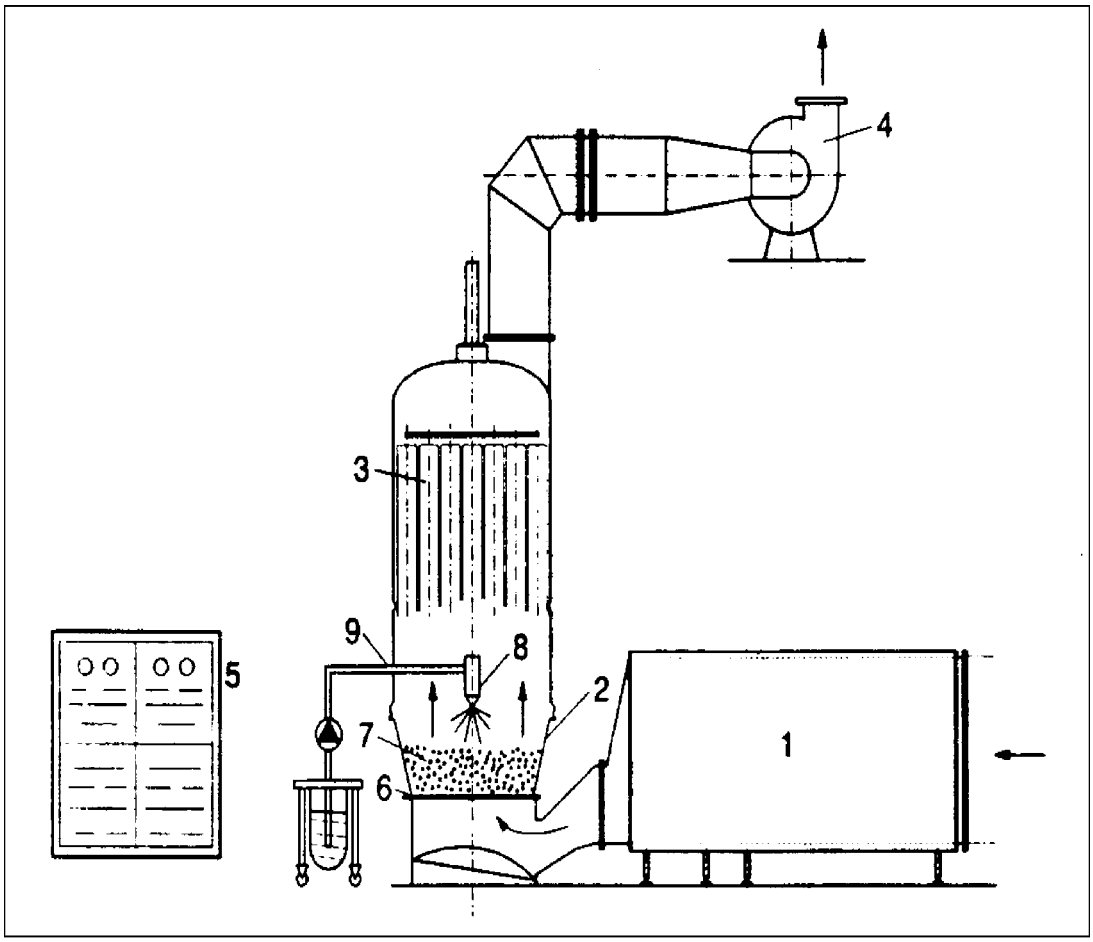

Figure 1. Fluid bed granulation schematic. This diagram illustrates the air and product flows during a representative granulation process (Courtesy of Pharmaceutical Technology (March 1991) article by Dilip Parikh entitled 'Airflow in Batch Fluid Bed Processing)'.

Analytical assays

Amino acids were quantified using the Waters AccQ-Tag precolumn derivatizing agent followed by a reverse-phase HPLC separation and fluorescence detection. Vitamins were assayed using a reverse-phase HPLC separation followed by UV detection. Osmolality was determined using an Osmette A (Precision Systems). Media pH values were obtained on a Mettler Toledo pH unit model MP220. Moisture content was determined using a Karl Fischer moisture apparatus following current USP procedure.

Results

Structure of AGT^{TM} granule

Figure 2 is a scanning electron photomicrograph of AGT^{TM} granulation. Granules of variable size result due to the action of wetting and then instantaneous drying of the media powder particles. Milled ingredient particles form a porous lattice-like structure that facilitates increased wetability, resulting in faster dissolution of AGT compared to standard dpm.

Biochemical data for AGT^{TM} VP-SFM

Osmolality and pH

Table 1 shows the non-adjusted pHs and osmolality values from three independent 9 kg pilot lots of VP-SFM in AGT^{TM} format reconstituted in production

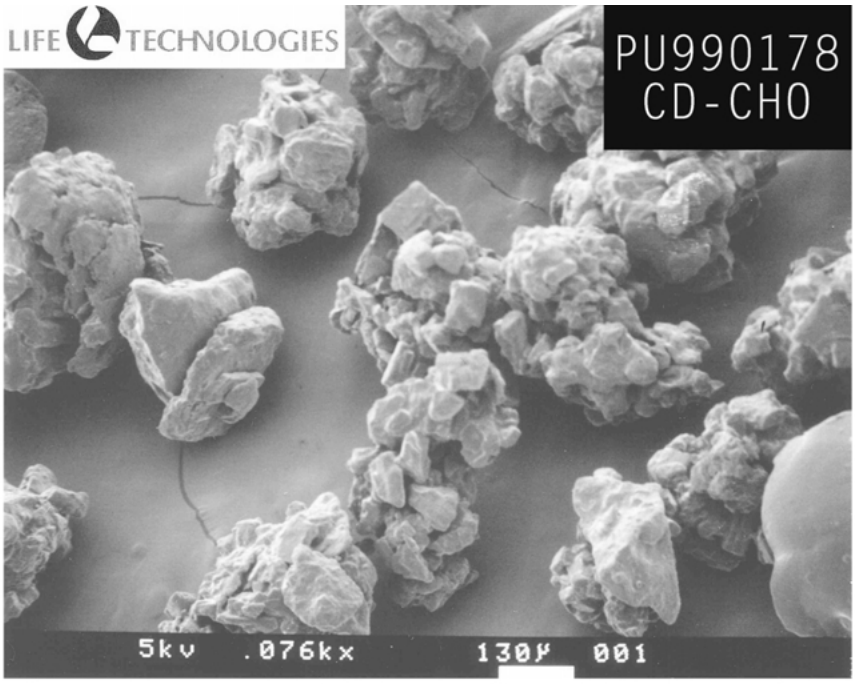

Figure 2. Photomicrograph of AGT CD CHO granules. This scanning electron photomicrograph (76X) illustrates the appearance of CD-CHO Medium prepared using the AGT^{TM} process. Milled constituent particles form a porous lattice-like structure (granule) that facilitates granule wetability and dissolution. (Photo courtesy of H. Vali, McGill University.)

Table 1. Biochemical analysis of AGT^{TM} VP-SFM

Lot number	pH units	Osmolality
AGT Lot A	7.28	304
AGT Lot B	7.29	301
AGT Lot C	7.29	302
Standard deviation	±0.0052	±1.665

Determination of pH and osmolality values for three pilot (9 Kg) lots of VP-SFM produced in the AGT^{TM} format.

grade water. pH values fell within a range of 7.28–7.30, with a standard deviation of ±0.0052, which is well within the tolerance of cells and the acceptable range for cell culture media[1]. The osmolality ranged 301–304 mOsm with a standard deviation of ±1.665, corresponding to an exceptionally tight distribution representative of a highly ordered and consistent process.

Homogeneity

A thorough homogeneity testing regimen was described elsewhere for AGT^{TM} CD-CHO (Radominski et al., 2001). Similar data are presented to confirm homogeneous distribution of various media constituents in granulated VP-SFM. Replicate samples were taken from multiple locations within the storage container for each of three test lots of VP-SFM. As observed (Figure 3), the AGT^{TM} test samples exhibited comparable precision for each analyte and compared well with the direct weigh reference control. Comparable data were also obtained for AGT^{TM} CD-Hybridoma, AGT^{TM} 293 and AGT^{TM} SF-900II (data not shown).

Cell growth and productivity

Cell growth and productivity data are presented for AGT^{TM} versions of VP-SFM, CD-Hybridoma, 293-II and SF-900II in Tables 2–5, respectively. Results are presented relative to performance of control media[2].

VP-SFM

Table 2 presents data for AGT^{TM} VP-SFM. Both Vero cell population doubling times and TCID50 ml^{-1} (bovine reovirus type 3) were equivalent for AGT^{TM} VP-SFM as compared to a catalogue control.

CD-Hybridoma

Murine hybridoma cell growth and monoclonal antibody (IgG) production were similar for both AGT^{TM}

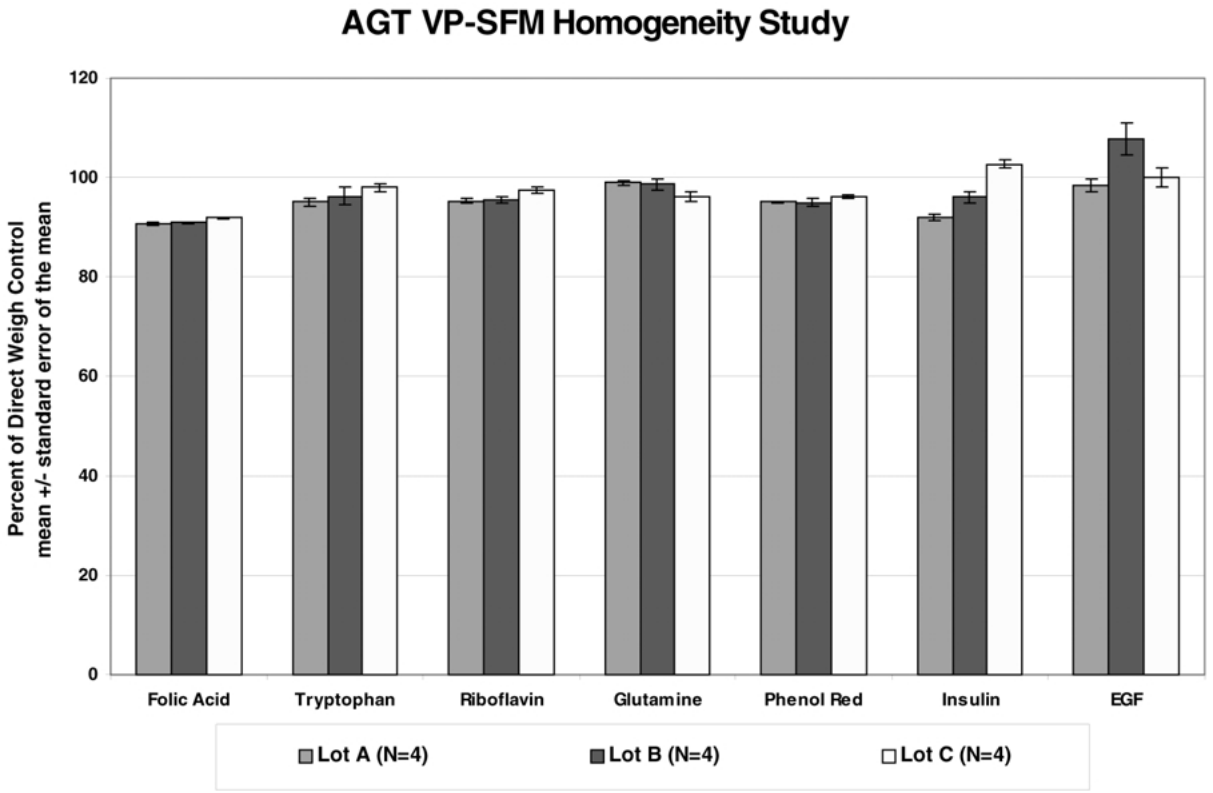

Figure 3. HPLC homogeneity evaluation. This homogeneity study demonstrated consistent distribution of representative analytes (folic acid, tryptophan, riboflavin, glutamine, phenol red, insulin, EGF) in three pilot lots (9 kg) of VP-SFM. Samples were prepared and analyzed as described in the section Materials and Methods.

Table 2. Biological performance of AGT^{TM} VP-SFM

Lot number	VERO cell growth	Virus production
AGT Lot 193	36.6	7.37
AGT Lot 194	34.9	6.88
AGT Lot 195	34.4	7.10
Direct weigh control	39.4	7.14

Biological performance of VP-SFM was analyzed in the AGT^{TM} format and compared with direct weigh controls. Vero cell growth is expressed as a population doubling time (hours). Virus production was determined using bovine reovirus, type 3, and expressed as $\log_{10}$ TCID50 ml^{-1} on day 5 post-infection.

Table 3. Biological performance of AGT^{TM} CD-hybridoma

Lot number	AE-1 growth	AE-1 productivity
Lot 203–001	1.82	45.46
Lot 203–002	1.73	46.00
Lot 203–003	1.81	50.38
Lot 238–002	2.20	49.99
Catalog Control	1.98	52.43

Biological performance of CD-Hybridoma Medium was analyzed in the AGT^{TM} format and compared with catalog controls. AE-1 (murine Sp2/0-derived hybridoma) cell growth is expressed as the maximal cell density achieved after 4 days (cells ml$^{-1} \times 10^6$). Productivity represents IgG production from a day 7 supernatant harvest (expressed as (μg ml^{-1}).

and control versions of CD-Hybridoma medium (Table 3).

293-II SFM

Table 4 shows results of HEK 293 cell growth and human Adenovirus Type 5 production. Cell growth in AGT^{TM} 293-II was slightly superior to direct weigh and catalogue controls (see Discussion). Adenovirus productivity was equivalent for all test samples.

Sf-900II SFM

Table 5 shows cell growth and plaque forming units (PFUs) productivity data for AGT^{TM} and both catalogue and a DPM control. Cell growth for all test

Table 4. Biological performance of AGT^{TM} 293-II SFM

Lot number	293 Cell growth	Doubling time	Virus production
AGT-2	3.21	33.89	9.28
AGT-3	3.30	30.15	9.18
Direct weigh control	2.09	42.23	9.00
Catalog control	0.94	42.23	9.16

Biological performance of 293-II SFM was analyzed in the AGT^{TM} format and compared with both catalog and direct weigh controls. HEK 293 cell growth is expressed both as the maximal cell density achieved on day 7 (cells $ml^{-1} \times 10^6$) and as population doubling time (hours). Virus production was monitored by infection of HEK 293 cells with human adenovirus, type 5, and expressed as $\log_{10}$ TCID50 ml^{-1}.

Table 5. Biological performance of AGT^{TM} SF-900 II SFM

Lot number	Sf9 growth	Productivity
Lot 111-00-002	4.56	2.25
DPM control	3.80	1.15
Catalog control	5.34	1.60

Biological performance of SF-900-II SFM was analyzed in the AGT^{TM} format and compared with both catalog and direct weigh controls. Sf9 cell growth is expressed as the maximal cell density achieved on day 7 (cells $ml^{-1} \times 10^6$). Productivity is monitored by quantitation of plaque-forming units (PFUs) and expressed as units ml^{-1} ($\times 10^9$).

media were comparable. Viral plaques exhibited some differences: The DPM control yielded only half the PFUs of the AGT^{TM} Sf-900II. The AGT^{TM} granulation process permits superior delivery of lipids and other constituents (compared to traditional formats), which may account for the increased plaque formation in AGT^{TM} Sf-900II.

Discussion

The AGT^{TM} process appears compatible with a broad range of serum-free, protein-free and chemically-defined nutrient media. This article illustrates performance equivalence of the AGT^{TM}-based format with identical formulations produced by conventional methods using various specialty media designed for key biotechnology applications. The AGT^{TM} process overcomes many disadvantages inherent to the ball-milling process for powdered medium and yields homogeneous granules that rapidly disperse in aqueous solution. AGT^{TM}-based media are a single component dry-form nutrient medium that minimizes dust generation, dissolves thoroughly, and represents a complete formulation that does not require addition of any extra components such as sodium bicarbonate, growth factors, insulin, iron carriers or hydrolysates. The fluid bed granulation process does not add any additional constituents to the medium, is adaptable to a broad range of catalog and customized formulations, and is designed to achieve a desired pH and osmolality specification without further titration. Final moisture content of AGT^{TM} media was within the specified range for dry powder media (2–3%, data not shown).

The AGT^{TM} media format has performed equivalently to reference media made with identical raw material constituents. Extended shelf life evaluation of dry-form media manufactured by the AGT^{TM} process is on-going. Preliminary analysis of biochemical composition and biological performance indicates that these materials are stable for at least one year under refrigerated storage conditions and for several months under controlled ambient storage (data not shown). In summary, we have always seen comparability or slight improvement in growth and productivity using the AGT^{TM} format, although additional studies will be required to clarify causal elements. Despite a slightly higher cost compared with conventional ball-milled powders, the speed and simplicity of AGT^{TM} medium reconstitution (simple addition of granules to water followed by rapid dissolution and filtration), coupled with its pharmaceutical grade manufacture, make it an attractive consideration for large-scale production of therapeutic biologicals.

Conclusion

We demonstrated manufacture of representative specialty media for mammalian and insect cell culture applications. Biochemical properties and biological performance of AGT^{TM}-based media were comparable to identical formulations prepared by conventional methods.

Acknowledgements

The authors thank Robert Keem for his analytical support and insight. The AGT^{TM} project team acknowledges project support and leadership provided by Mark Plavsic and William Biddle. We are grateful to our colleagues in R&D for their technical assistance and suggestions, to Tinamarie Lovullo for manuscript

preparation, and to Desiree Payan for operational assistance and significant contributions.

Notes

[1] The AGT™ manufacturing process permits targeting the non-adjusted pH of reconstituted media to any desired pH.

[2] Catalogue control represents a 1X sample from inventory. Direct weigh control indicates a reference sample made with the same raw materials and at the same time as the AGT™ media.

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