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Formulation of Cell-Based Medicinal Products: A Question of Life or Death?

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ABSTRACT

The formulation of cell-based medicinal products (CBMPs) poses major challenges because of their complexity, heterogeneity, interaction with their environment (e.g., the formulation buffer, interfaces), and susceptibility to degradation. These challenges can be quality, safety, and efficacy related. In this *commentary* we discuss the current status in formulation strategies of off-the-shelf and non-off-the-shelf (patient-specific) CBMPs and highlight advantages and disadvantages of each strategy. Analytical tools for the characterization and stability assessment of CBMP formulations are addressed as well. Finally, we discuss unmet needs and make some recommendations regarding the formulation of CBMPs.

Introduction

Cell-based medicinal products (CBMPs) belong to an innovative and heterogeneous group of medicines based on cells or tissues, which may be *ex-vivo* genetically modified with, e.g., a viral vector (gene therapy medicinal products) and/or combined with a medical device, e.g., a biodegradable scaffold (combined CBMPs).[1] Most CBMPs contain live human (stem) cells, derived from either the patient (autologous) or a healthy donor (allogeneic). Since the late 1960s stem cells have been used in medical practise to treat blood cancers (hematopoietic stem cell transplantations (HSCTs)). It took another twenty-five years before the true value of stem cells and their therapeutic potential was explored and only since the beginning of the 21st century there has been a steep increase in the number of CBMP clinical trials. Currently, worldwide there are hundreds of trials ongoing for a great variety of indications. However, only a handful products have been approved so far and even fewer products are commercially successful. In Europe, for example, four out of eleven licensed CBMPs have been withdrawn from the market for commercial reasons and/or lack of clinical relevance (see Table 1).[2]

Recently, Bak and colleagues (2019) wrote: "We hope to assemble —in the Journal of Pharmaceutical Sciences— manuscripts addressing various aspects of lessons learned and discuss potential solutions for this emerging area of complex gene and cell-based therapeutic product manufacturing, development, …".[3] This commentary addresses the issue of CBMP formulation development.

Medicines tend to become more complex over time. Forty years ago, breakthroughs in molecular biology led to a surge of protein-based medicines: complex, 'fragile', three dimensional structures undergoing a multitude of chemical and physical degradation reactions. Nowadays, the formulation scientist of pharmaceutical proteins can use insights gained over the past forty years and design stable protein product formulations that are well-characterized by novel analytical means. As compared to these pharmaceutical protein products, CBMPs offer a slate of additional

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Table 1
Approved CBMPs in the EU (2008–2019).

Product (Classification); INN	Administration Route/Technique	Pharmaceutical Form; Drug Product Description	Shelf-Life and Storage Condition
ChondroCelect (TEP[a]) *Withdrawn*	Implantation into knee	Cell suspension; 4×10^6 viable autologous cartilage cells/0.4 mL DMEM[b] in 1 mL type I glass vial with chlorobutyl stopper and aluminum seal	48 hours at 15–25 °C
MACI (TEP) *Withdrawn*	Implantation into knee	Implantation matrix; $0.5–1 \times 10^6$ viable autologous chondrocytes/cm^2 on a 14.5 cm^2 CE marked porcine derived Type I/III collagen membrane in 18 mL solution (DMEM + HEPES; pH adjusted with HCl or NaOH; osmolality adjusted with NaCl)	6 days below 37 °C
Provenge (SCTMP); sipuleucel-T; *Withdrawn*	Intravenous infusion	Cell suspension; $\geq 50 \times 10^6$ autologous activated $CD54^+$ cells/250 mL buffer (NaCl, sodium lactate, KCl, $CaCl_2$) in breathable polyolefin tri-laminate bag	18 hours at 2–8 °C
Holoclar (TEP)	Implantation under conjunctiva	Living tissue equivalent & transparent circular sheet; $0.3–1.2 \times 10^6$ viable autologous human corneal epithelial cells containing stem cells ($79 \times 10^3–16 \times 10^3$ cells/cm^2) attached on a supportive 2.2 cm diameter fibrin matrix (containing thrombin, fibrinogen and aprotinin) in DMEM supplemented with L-glutamine in a screw-cap container	36 hours at 15–25 °C
Strimvelis (*ex-vivo* GTMP)	Intravenous infusion	Cell suspension; $1–10 \times 10^6$ transduced autologous $CD34^+$ cells/ml saline in one or more sterile EVA bag(s) with a Luer spike interconnector closed with Luer-lock cap	6 hours at 15–30 °C
Zalmoxis (*ex-vivo* GTMP) *Withdrawn*	Intravenous infusion	Cell suspension; $5–20 \times 10^6$ allogeneic transduced T cells/ml in 10–100 mL solution (containing NaCl, 10% DMSO, 7% HSA) in 50–500 ml EVA bag	18 months in vapor phase of liquid nitrogen
Spherox (TEP)	Intraarticular (into knee) implantation	Cell suspension; ≤ 100 spheroids (spherical aggregates of autologous chondrocytes associated to extracellular matrix) in ≤1000 μL saline in syringe (polypropylene; Luer-lock, isoprene sealing ring, and cover cap) or ≤ 60 spheroids in ≤200 μL saline in applicator (polyurethane catheter, acrylonitrile butadiene styrene sealing plug on one side and silicone stopper on the other side)	72 hours at 1 °C–10 °C
Alofisel (SCTMP); darvadstrocel	Intralesional injection	Cell suspension; 30×10^6 allogeneic mesenchymal adult stem cells in 6 ml solution (DMEM + 20% HSA) in 9 ml Type I glass vial with rubber stopper and flip-off seal	72 hours at 15–25 °C
Kymriah (*ex-vivo* GTMP); tisagenlecleucel	Intravenous infusion	Cell suspension; $1.2 \times 10^6–6 \times 10^8$ anti-CD19 CAR^+ genetically modified autologous viable T cells in 10–30 ml solution (glucose; NaCl; HSA; dextran 40 for injection (10 mg/ml); DMSO (82.5 mg/mL); Na-gluconate; Na-acetate; KCl; $MgCl_2$; Na–*N*-acetyltryptophanate; Na-caprylate; aluminum; water for injection) in 50 ml EVA bag(s) with PVC tubing and Luer spike interconnector closed by Luer-lock cap, or 30–50 mL in 250 mL bag	9 months at below −120 °C in vapor phase of liquid nitrogen
Yescarta (*ex-vivo* GTMP); axicabtagene ciloleucel	Intravenous infusion	Cell suspension; $0.4–2 \times 10^8$ anti-CD19 CAR^+ genetically modified autologous viable T cells in ca. 68 mL solution (CryoStor CS10[d]; NaCl; HSA) in EVA bag with sealed addition tube and two spike ports	1 year at below −150 °C in vapor phase of liquid nitrogen
Zynteglo (*ex-vivo* GTMP); betibeglogene autotemcel	Intravenous infusion	Cell suspension; $1.2–20 \times 10^6$ autologous genetically modified $CD34^+$ cells/ml solution (CryoStor CS5[d] and NaCl) in 20 mL fluorinated ethylene propylene cryopreservation bag	1 year at below −140 °C in vapor phase of liquid nitrogen

[a] Abbreviations: CAR = chimeric antigen receptor; DMEM = Dulbecco's modified Eagles medium; DMSO = dimethyl sulfoxide; EVA = ethylene vinyl acetate; GTMP = gene therapy medicinal product; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSA = human serum albumin; PVC = polyvinyl chloride; SCTMP = somatic cell therapy medicinal product; TEP = tissue engineered product.

[b] DMEM contains: $CaCl_2$, $Fe(NO_3)_3$, KCl, $MgSO_4$; NaCl, $NaHCO_3$; KH_2PO_4; glucose, L-cystine.2HCl, L-glutamine, glycine, L-histidine.HCl, L-isoleucine, L-leucine, L-lysine.HCl, L-methionine, L-phenylalanine, L-serine, L-threonine, L-tryptophan, L-tyrosine.2Na, L-valine, D-Ca-pantothenate, choline chloride, folic acid, i-inositol, niacinamide, riboflavin, thiamine.HCl, pyridoxine.HCl.

[d] CryoStor CS5/CS10: proprietary formulation containing 5%/10% DMSO, respectively, dextran-40, sodium, potassium, calcium, magnesium, phosphate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), lactobionate, sucrose, mannitol, glucose, adenosine, and gluthathione.

challenges to the formulation scientist. The inherent complexity of CBMPs, as compared to classical biopharmaceuticals, poses unique quality (chemical, manufacturing, and controls (CMC)) questions. In Table 2 the main CMC differences between CBMPs and pharmaceutical proteins —the largest group of established biopharmaceuticals— are listed. In order to consistently manufacture live cells of high quality that are safe and efficacious, a thorough understanding of the desired cell characteristics and the interaction of cells with their environment during processing, storage, transportation, handling and administration is key. This should come with extensive process and product characterization, which is a challenging undertaking because of the lack of sensitive, stability-indicating, orthogonal and complementary analytical techniques. This is also true for a full understanding of critical quality attributes (CQAs). However, high variability of starting materials, high process variability, limited availability of material for in-process and drug product testing, are challenges the field currently faces. For instance, for autologous CBMPs no patient material can generally be procured at all for formulation development. Surrogate material (e.g., allogeneic starting material, relevant cell lines, or cadaver cell material) is being used to gain information on formulation and stability strategies. This approach obviously bears the risk that it is not fully representative for autologous clinical materials. Where allogeneic material from a healthy donor can be obtained, low quantities remain a challenge, since for most cell types material from various donors cannot be pooled and their expansion capabilities to increase the batch size for extensive drug product testing using the desired clinical product presentation are limited.[4] Exceptions are pluripotent stem cell-based CBMPs, for which master and working cell banks can be created.

The aim of formulation development is achieving a high-quality, stable medicinal product that is safe and efficacious and meets regulatory requirements. The focus therefore is multi-factorial and includes stabilization of the cellular active pharmaceutical ingredient (API), so that it can withstand stress factors, such as temperature excursions, e.g., freeze-thawing, mechanical stress, oxidative stress, CO_2 stress, pH changes and contact with interfaces, which it may encounter during fill & finishing, storage, shipment, preparation for administration and administration to the patient.[4,5] Other key aspects are the selection of excipients of pharmaceutical grade, manufactured according to GMP principles, and assurance that the formulation step is fully integrated in the aseptic manufacturing process and minimizes the risk of microbial contamination of the CBMP. Obviously, sterility and low endotoxin levels of the excipients are a must.

Up until now, however, rational design of the formulation for CBMPs, based on the requirements defined in the (quality) target product profile ((Q)TPP), has received relatively little attention.[6–8] This is illustrated by the short CMC sections on formulation development in regulatory filings for marketing authorization: a few pages for a CBMP versus over a hundred pages for a monoclonal antibody product (KHH, personal observations). Furthermore, a search using the key words 'formulation' AND 'cell-based medicinal products' (Scopus, TITLE, April 1, 2020) gives no hits. An important reason for this is that these products are historically mostly developed by medical scientists in hospital settings using different terminology than their colleagues working on classical biopharmaceuticals (see Table 3). Moreover, the composition of CBMP formulations and their storage conditions are historically often copied from HSCT products. This paradigm has been changing over

Table 2
Pharmaceutical Characteristics of CBMPs as Compared to Protein Pharmaceuticals.

Characteristic	CBMP	Protein Pharmaceutical
API[a]	Cell (may or may not be genetically modified)	(Glyco)protein
Size API	~10 μm	~10 nm
Live material	Yes	No
Mode of action	Partly unknown, complex; contributions of cells (e.g., cytokines) and particles (e.g., exosomes)	Known; interaction with receptor(s)
Product and process impurities	Cellular impurities (e.g., debris, unwanted cell populations); process impurities (e.g., beads used for activation, FCS, growth factors, antibiotic)	Protein degradants; process-derived impurities (e.g., host cell proteins, DNA, column materials)
Stability	Susceptible to changes in pH, osmolality; cell culture components; temperature; freeze-thawing; mechanical stress; necrotic or apoptotic cells (DNases); cryoprotectants (DMSO)	Susceptible to degradation due to changes in pH, temperature; mechanical stress; light and oxidative stress; freeze-thawing
Manufacturing process	Often continuous, no designated DS	Often discontinuous process, designated DS and DP
	Often open and manual aseptic process steps; no platform technologies yet; automation in its infancy	Closed and mostly automated process steps; platform technologies
	No viral removal and/or inactivation steps; final sterilization not possible	Viral removal and/or inactivation steps; final sterile filtration
Characterization	Limited set of established stability-indicating analytical tools	Multiple established stability-indicating analytical tools
QC testing	Full QC testing not always possible prior to product administration; retention samples for retesting not always possible	Full QC testing performed prior to product administration; retention samples for retesting available
Formulation composition	Multiple components	Few components
Container & closure system	Infusion bag; vial & stopper & cap	Vial & stopper & cap; pre-filled syringe
Shelf-life and storage conditions	Hours or days in liquid form (2–8 °C, room temperature or 37 °C); up until years in frozen form (below −120 °C or −150 °C)	2–3 years at 2–8 °C in liquid or lyophilized form
Handling and administration	In clinic; standard procedures not always available	In clinic or at patient's home; standard procedures available
Route of administration	Predominantly intravenous	Intravenous; subcutaneous; intramuscular
In vivo half-life	Minutes to years	Minutes to weeks
Clearance mechanism	Trapped primarily in spleen, liver, lung	Enzymatic degradation; hepatic and/or renal clearance
Batch definition	Often one batch for one or few patients (non-off-the-shelf); off-the-shelf products less common	Off-the-shelf (one batch for multiple patients)
Batch consistency	Low: autologous non-off the shelf; medium: allogeneic off-the shelf	High

[a] Abbreviations: API = active pharmaceutical ingredient; DMSO = dimethyl sulfoxide; DP = drug product; DS = drug substance; FCS = fetal calf serum.

Table 3
Pharmaceutical Terminology Used for Classical Biopharmaceuticals Versus CBMPs.

Classical Biopharmaceuticals	CBMPs
Aggregate	Cell clump
Excipients	Additives
Formulation buffer	Composition, cryopreservation medium, biopreservation medium, solution
Frozen liquid storage	Cryopreservation
In-use stability	Handling in clinic
Liquid formulation	Fresh cell suspension
Medicinal product for human use	(Stem) cell therapy; regenerative medicine
Real-time stability condition, storage	Preservation
Shelf-life	Durability
Shipment, administration	Delivery

the last decade and, as will be demonstrated below, the wish list for better CBMP formulations is long.

In the following, we first discuss the typical stability issues that apply to CBMPs. Next, we report on the current status and unique aspects of formulating CBMPs, including current formulations, typical excipients used and strategies followed. Advantages and limitations of each strategy are addressed in light of different therapeutic concepts, e.g., a single autologous or allogeneic product batch to treat an individual patient (non-off-the-shelf) or a large batch of an off-the-shelf allogeneic product to treat several patients. These two different product categories come with different manufacturing technologies and stability requirements. Furthermore, analytical tools for the characterization of CBMP formulations and stability testing are highlighted and unmet analytical needs are discussed. Finally, conclusions and recommendations for future directions are presented.

Stability and Degradation Pathways of Cells

In this paper, cell stability is referred to as a combination of physical, chemical and biological, including genotypic and phenotypic, stability. Physical stability is defined as the property of the cell to remain a single-cell dispersion. Due to different stress factors (discussed later) cells may form clumps or even bigger agglomerates, especially at high cell concentrations (1×10^8 cells/mL).[4] These may sediment and cause cell death or problems with parenteral administration, such as clogging of needles. Chemical degradation may occur when compounds inside or outside the cell (part of the formulation) undergo chemical changes, such as oxidation and hydrolysis of proteins and lipids. Biological stability is defined as the property of the cell to remain viable, healthy, and potent and hence can elicit a biological activity upon administration to humans. It also entails a stable cell identity, as assessed by genotypic and phenotypic characteristics.[9]

In contrast to traditional biopharmaceuticals, cells are viable entities. They have an active metabolism, eat (phagocytosis), drink (pinocytosis), secrete active molecules and shed particles (e.g., exosomes) in their environment and communicate with neighboring cells through various mechanisms. Cells may lose their integrity via different pathways, including apoptosis and necrosis.[10] Degrading cells can secrete DNase and other lytic enzymes, causing instability of neighboring cells. Dying cells become cell debris and can form clumps, which may induce physical instability and consequently initiate biological instability since cells in the center of a big aggregate can easily die because of lack of oxygen. Hence, physical, chemical, and biological instability may go together, and it is not always obvious which of those is the major driver causing instability. On the other hand, cells have strategies to protect themselves against instability. Evidently, once in the frozen (cryopreserved) state, cell metabolism is minimized, hence cell degradation is minimal. Freezing and thawing processes, however, may stress the cells and cause physical and biological instability (e.g., early and late onset of cell death).[4,5] Therefore, it is critical to carefully develop optimal freezing and thawing protocols and use appropriate equipment for these steps.[11]

Formulation of CBMPs

Current Formulations and Storage Conditions: Fresh or Frozen

Currently, there are two main categories of CBMP formulations: non-frozen liquid (fresh) and frozen liquid (cryopreserved) cell suspensions (cf. Table 3). Most of these formulations are complex, i.e., they contain many ingredients. Seven of the eleven CBMPs approved in the EU are non-frozen liquid formulations. These contain either Dulbecco's Modified Eagle Medium (DMEM, a cell culture medium with about 30 components) or a simple buffer, are stored at room temperature (five) or in the refrigerator (two) and have a shelf-life of hours to days (Table 1). The other four CBMPs are frozen formulations that contain dimethyl sulfoxide (DMSO) and human serum albumin (HSA). They are stored at < -120 °C in the vapor phase of liquid nitrogen (i.e., cryopreserved) and have a shelf-life of months to years (Table 1). Other compounds, such as glycerol, ethylene glycol, dextran, hydroxycellulose, lactobionic acid, disaccharides and mannitol, enhance cell stability when combined with DMSO. In addition, most frozen liquid formulation buffers contain amino acids, vitamins and trace metals to supply nutrients to the cells.

The formulations and shelf-lives of fresh and frozen CBMPs in clinical development present a similar picture.[12] Frozen T-cell products are predominantly formulated in 5–10% DMSO, HSA (5–20%) and an isotonic multi-electrolyte solution, such as PlasmaLyte A. Dendritic cell (DC) and natural killer (NK) cell formulations stored under frozen conditions are comparably complex. Some products contain CryoStor CS5 or CS10, a proprietary, 'ready to use', complex medium of more than fifteen excipients containing 5% and 10% DMSO, respectively (see Table 1). All components present in CryoStor CS5 and CS10 are of chemical origin and comply with compendial standards for pharmaceutical excipients, except for HEPES and lactobionic acid. The CryoStor CS solutions are manufactured according to GMP standards and extensively tested.[13] Notably, several CBMPs in clinical development contain fetal calf serum (FCS) or human plasma as stabilizer. Some, especially DC-based frozen products, need additional handling in the clinic, such as a post-thaw wash to remove the DMSO followed by an additional cell culture step. Commonly used excipients in CBMP formulations are listed in Table 4. Except for cryoprotectant agents (CPAs), ingredients found in frozen CBMP formulations are also encountered in non-frozen formulations (see Table 1).

There is limited information in the public domain about the rationale for selecting a specific formulation and storage temperature. DMEM has been used as cell culture medium for decades. Developers of non-frozen CBMPs may have taken a 'pragmatic'

Table 4
Common Excipients in CBMPs.

Excipient Class	Function	Examples
Buffer	pH control; tonicity	Tris, histidine, sodium acetate
Salt	Tonicity; stabilization	Sodium chloride, potassium chloride, magnesium chloride
Sugar[a]; polyol	Tonicity; stabilization; cryoprotection (extracellular CPA[b])	Trehalose, sucrose, glucose (reducing sugar), penta-isomaltose, mannitol
Polysaccharide	Collapse temperature modifier (extracellular CPA)	Dextran, hydroxyethyl starch
Amino acid	Stabilization; tonicity; pH control; cryoprotection	Multiple; e.g., DMEM components (see Table 1)
Nucleoside	Stabilization	Adenosine, guanosine
Antioxidant	Oxidation prevention	Methionine, sodium edetate
Organic solvent	Cryoprotection (intracellular CPA)	Glycerol, ethylene glycol, DMSO
Protein; polypeptide	Stabilization; cryoprotection	HSA, FCS, human plasma, poly-L-lysine

[a] Preferably non-reducing.
[b] Abbreviations: CPA = cryoprotectant agent; DMEM = Dulbecco's modified Eagle's medium; DMSO = dimethyl sulfoxide; FCS = fetal calf serum; HSA = human serum albumin.

approach to choose this cell culture medium as storage and transport medium. However, it is questionable if all the medium components are required, or favorable, for cell stability: culture media have been optimized to promote cell growth, not to preserve cell quality during storage and shipment. Moreover, cell culture medium ingredients are not necessarily of pharmacopeial grade and manufactured according to appropriate GMP standards. Excipients such as DMSO, dextran 40, FCS, human plasma and HSA may pose various safety risks to the patient. For instance, human plasma and HSA may carry the risk for transmission of blood-borne contaminants.[14] Moreover, it has been shown that the presence of DMSO may destabilize HSA during freezing and thawing.[15] Other potential problems are related with reliability of supply, batch-to-batch consistency and country-specific requirements, which does not allow for a universal global product. Dextran may induce anaphylactic reactions.[16] Issues with DMSO are discussed in the following section.

Frozen Liquid Formulations

Since non-frozen cells are short lived, cryopreservation has for decades been the solution to cell stabilization (Table 1).[17] However, the freezing and thawing processes will cause cell death unless stabilizing excipients are included in the formulation. The mechanism of action of CPAs is complex and is not completely understood. Importantly, frozen CMBP formulations typically include a so-called penetrating (or intracellular) CPA, which readily passes the cell membrane to suppress ice formation not only outside but also inside the cells. The most widely used intracellular CPA is DMSO. Besides DMSO and/or other intracellular CPAs (see Table 4), the formulation may contain non-penetrating (extracellular) CPAs, such as sucrose, trehalose and dextran, whose mechanism of action is thought to be related at least in part to their stabilizing interaction with cell membranes.

Frozen CBMPs are predominantly stored in the vapor phase and sometimes in the liquid phase of liquid nitrogen tanks. These tanks maintain a temperature of about −196 °C, the boiling point of liquid nitrogen. Sometimes extra cold freezers (−80 °C to −150 °C) are used. However, long-term storage at a temperature above −120 °C is for most CBMPs used in the clinic to date a semi-stable condition, since the glass transition temperature of currently used formulations is generally lower than −100 °C.[18] Storage under ultra-low temperatures (called cryopreservation or cryostorage) allows for a product stability of months to years, since it reduces cell metabolism to almost zero activity. The cryopreservation process entails the following general unit operations, all of which may induce cell instability and thus need to be studied during formulation studies: 1. cell harvesting; 2. addition of ready-to-use cryopreservation formulation buffer and/or CPAs and other excipients; 3. the freezing procedure; 4. long-term storage under frozen conditions, especially if these conditions are not properly maintained; 5. the thawing procedure; 6. if needed, cell washing prior to administration to remove DMSO and/or excipients of biological origin, such as FCS.[4,19]

The main advantage of cryopreservation is a long product shelf-life, which allows for full quality control (QC) release testing of the product prior to administration. Moreover, the manufacturing can be scheduled independently of patient pretreatment conditioning. In this case the product transport and preparation for administration can be performed on demand, when the patient is ready to receive the cells. In addition, a central production site is possible, a model proven to be successful for conventional medicines. Furthermore, the same product batch can be used for repeated dosing. However, there are also disadvantages of frozen storage, as discussed below in more detail for DMSO, and freeze-thaw induced cell stress. Furthermore, freezing, long-term storage, transport and thawing under controlled conditions require complex, expensive equipment. Many clinical centers, especially in developing countries, do not have access to or cannot afford such equipment. Another drawback is that cells may need 12−24 hours to recover from the freeze-thaw stress to retrieve full functionality, which may not be practical for various reasons.[20]

CBMPs and DMSO: A Love-Hate Relationship

All EU marketed frozen CBMPs (Table 1) and a lot of frozen CBMPs in clinical development contain 5−10% DMSO as intracellular CPA.[12,13] While needed to protect the cells against freezing stress, DMSO in these concentrations is toxic to cells in the non-frozen state. This implies that pre-freeze and post-thaw CBMP stabilities in the presence of DMSO are short. Furthermore, DMSO has to be added to the cells gradually under chilled conditions to prevent cell instability resulting from osmotic stress and heat-shock, respectively.[21] For an off-the-shelf CBMP batch of hundreds of vials, fill & finishing and freezing has to be well coordinated to avoid vial-to-vial variability due to different times cells are exposed to DMSO prior to freezing. Another disadvantage of DMSO is its incompatibility with various production systems, disposables (tubing), primary packaging materials and administration devices, generating DMSO-induced leachables.[22] For instance, plasticized polyvinyl chloride (PVC) is commonly used for manufacturing tubing as well as IV tubing in hospitals; PVC and its plasticizers such as di(2-ethylhexyl) phthalate (DEHP) are readily dissolved in DMSO, which potentially results in toxic effects.[23]

Given its cellular toxicity, it is not surprising that DMSO also causes adverse effects in patients. Although DMSO is classified as solvent with a low toxic potential (i.e., a <50 mg/day intake is acceptable[24] and intravenous administration up to 1 g/kg/day is common practice in the transplant world,[25,26] DMSO depletion reduces the frequency of adverse effects.[27] Infusion of DMSO-

preserved cells into patients is often associated with toxic reactions such as nausea, vomiting, cardiac dysfunction, anaphylaxis, acute renal failure, hypotension and transient hypertension.[28] Therefore, patients are generally pre-medicated with paracetamol and intravenous chlorphenamine prior to the infusion of DMSO-containing CBMPs to mitigate the possible adverse effects. However, these pre-medications don't protect from another adverse effect: DMSO makes patients smell like garlic because of the formation of sulfur-containing metabolites. This is not only very inconvenient for the patients but it also has to be considered when setting up (double) blind clinical trials.

Because of the above concerns with DMSO, some CBMPs undergo washing and concentration steps prior to administration.[12] This is not always possible, or even desirable, at the clinical site. Additional product handling steps in the clinic bear a risk of contamination and may compromise CBMP stability.

Primary Packaging

Although not directly related to formulation, compatibility of CBMP formulations with the primary packaging materials needs to be assessed. Depending on the route of administration, cell concentration and filling volume, either cryobags (50–500 ml) or cryovials (1–50 mL) are selected as container closure systems for frozen CBMPs. The majority of bags currently available in the market are primarily made of ethyl vinyl acetate or another copolymer. Since these copolymers become brittle under cryogenic conditions, a metal cassette as secondary packaging is used during transport of the cell bags. Cryovials are composed of cyclic olefin co-polymer, the stopper of a thermoplastic elastomer, and the seal of aluminum, and are more break resistant under cryogenic conditions.[4] However, demonstrating container closure integrity for vials under cryogenic conditions remains challenging.[29]

Non-Frozen Liquid Formulations

Advantages of non-frozen (fresh) CBMPs include, amongst others, absence of freeze-thaw related cell instability and consequently, absence of CPAs such as DMSO, and a more straightforward and cheaper supply chain. Moreover, there are less issues with primary packaging materials as compared to frozen products. Some containers are the same as for conventional parenteral products, such as type I glass vials and polypropylene syringes (see Table 1). It has been shown for various CBMPs, such as mesenchymal stem cell-derived products, NK products and T cell products, that fresh products have a significantly higher purity, viability and potency than frozen products.[30–32]

Downsides of non-frozen storage conditions are that a production site close to or at the treatment center is necessary for multi-country trials and for global commercial product supply. Moreover, full QC testing prior to product administration often is not feasible, as some tests take several weeks, which is longer than product shelf-life (a few hours to several days; see Table 1). This implies that the product is administered at risk. Finally, a tight coordination between the manufacturing facility, shipment company, health care providers and the patient is a must.

CBMP Formulation Development: Quo Vadis?

Time for Rational Formulation Design

Although there is a clear trend towards the development of more economic off-the-shelf allogeneic CBMPs to treat larger patient populations with one batch, there are still a lot of non-off-the-shelf products in the pipeline for personalized therapies. Therefore, we now focus on strategies for the selection of a formulation for both modalities to achieve a high-quality, safe and efficacious product. Product specific design parameters, such as cell dose, wanted and unwanted cell (sub-)populations, primary packaging material(s), storage, shipment and administration must be considered during formulation studies. In addition, down-stream production process steps have to be taken into account. It seems unlikely that one optimal –generic– formulation for such a heterogeneous group of CBMPs will be found: no 'one size fits all' for CBMP formulations.

Off-The-Shelf Products

Extensive formulation development studies should be carried out for these medicines and alignment with big pharma drug development (i.e., formulation & stability) experiences and strategies for other off-the-shelf biopharmaceuticals is warranted. This would also include biotech industry experiences with regulatory documents and formulation development related content in these filings. Approaches for developing improved formulations to allow for long shelf-life and global distribution are discussed in this section.

To assure that CQAs of the CBMP are maintained from storage throughout the supply chain up to administration to the patient, the selection of the most appropriate excipients in optimal quantities is key. Specifically, the search for formulations with safe and/or chemically well-defined alternatives for DMSO, HSA and some other components currently used in frozen product formulations is critically important. Here, the extensive knowledge available in the field of fundamental cryobiology of animal, human and plant cells as well as tissues and relevant expertise of pharmaceutical scientists could be of help for the CBMP formulation scientist to rationally design the formulation. Suitable alternatives for DMSO may include extremolytes, such as ectoine and its derivatives, as well as other osmolytes. Ectoines are small molecules that are accumulated in extremophiles as osmoprotectant solutes. These cyclic amino acid derivates do not interfere with cell metabolism. Promising results have been demonstrated for frozen NK-cells.[33] Improved post-thaw NK-92 cell activity was obtained in a formulation containing poly-L-lysine, ectoine and dextran. For Jurkat cells, used as model for $CD3^+/CD4^+$ T-lymphocytes, frozen in a formulation of sugars, amino acids and sugar alcohols, a high post-thaw viability was observed.[34] DMSO-free fully chemically defined proprietary ready-to-use formulation buffers are also available, although not extensively tested or clinically used to our knowledge.[35]

Lyophilization

Lyophilization, or freeze-drying, is not only commonly used for stabilizing protein drug products,[36] but also has been shown as a means to increase the shelf-life of plasma and vaccines,[37] blood platelets,[38] and sperm cells.[39,40] Moreover, and highly relevant in this context, a few groups have reported on the freeze-drying of mammalian cells (including CBMPs)[41–48] and whole blood.[49] The availability of freeze-dried CBMPs with a long shelf-life at ambient or refrigerated conditions would mitigate a lot of the current issues with storage and logistics of off-the-shelf products worldwide. Therefore, it would make sense to make resources available for exploring the feasibility of lyophilized CBMPs. Evidently, this should include the search for suitable lyoprotectants, such as trehalose, to protect proteins, cellular membranes, nucleic acids and mitochondria against the harmful effects of dehydration.[50–54]

Non-Off-The-Shelf Products

For non-off-the-shelf CBMPs, formulation development should target for non-frozen liquid (fresh) storage, since this comes with a lot of advantages, such as simplification of production processes. Especially when fully closed and automated production systems are used and integrated with closed formulation and fill & finishing steps, batch-to-batch inconsistency and comparability may become less of an issue. This requires a production site nearby the treatment sites,

resulting in short transport times. Even bedside production may be feasible in the near future.[55] For these cell products the shelf-life is dictated by QC timelines and patient conditioning schedules. Since DMEM and other cell culture media may not be appropriate buffers for cell formulation and storage, simpler buffer solutions containing less excipients should be the primary focus of formulation development. Replacement of human and animal derived excipients, e.g., by recombinant HSA or chemically defined excipients, is key. Ideally, formulation improvements should lead to shelf-life extension, preferable under non-refrigerated conditions. Possibly, natural deep eutectic solvents (NADES), composed of liquid mixtures of several solid compounds, such as sugars, polyols, amino acids and aliphatic acids, might offer —literally— solutions to the problem.[56]

If a 'fresh' formulation strategy is not feasible or desirable, e.g., when repeat dosing is required or patient conditioning may need to be adjusted to patient's health situation, a frozen liquid storage has to be considered. In that case, the formulation strategies discussed above for off-the-shelf CBMPs can be followed.

Analytical Methods

Current Status

Table 5 lists analytical techniques that are currently applied to assess CBMP quality. Analytical tools measuring cellular impurities, viability, dose, potency, general attributes and sterility are part of process and product characterization, QC testing and formulation and stability studies. Although Table 5 illustrates that we can measure a lot of quality attributes, few of the current analytical techniques are both sensitive, precise and stability-indicating. Poor precision may explain in part why for many QC methods wide acceptance criteria are set (e.g., viability by nucleocounter > 70%; purity by flow cytometry > 60%; potency by ELISA 10× higher than background). A key question when evaluating the results of these and other methods for formulation development is: is the product really stable, or are the methods used unable to show instability? The limitations of our current analytical arsenal are one of the reasons why CBMP formulation development is so challenging, as will be illustrated in the examples below.

Viability Assays

Although a decline of cell viability is indicative of CBMP instability, classical viability assays, such as the trypan blue exclusion assay, only tell us if a cell is 'live' or 'dead', but not if a living cell is on its way to die. So, a viability assay may be stability-indicating, but may lack sensitivity for detecting early-stage degradation. More advanced cell death assays may be more sensitive.[10] For instance, flow cytometry can be used to distinguish between early-stage apoptotic, late-stage apoptotic and necrotic cells.[5,11,57,58] However, measuring early and late onset of cell death by flow cytometry is

Table 5
Analytical Tools Used for CBMP Characterization and Their Stability-Indicating Power.

Quality Attribute	Examples & Techniques	Stability Indicating
Identity		
	Phenotypic markers by flow cytometry	Potentially
	Transgene expression by qPCR[a]	Potentially
	Genetic identity by STR analysis	Potentially
	Karyology by microscopy	Potentially
Viability (total, viable and dead cells)		
	Automatic cell counting (several fluorescence- and dye-based techniques)	Yes
	Trypan blue exclusion test (microscopic evaluation)	Yes
Strength/dose		
	Viable cell concentration by automatic cell counting	Yes
Purity & cellular impurities		
	Phenotypic markers by flow cytometry	Potentially
	Pluripotent stem cells by qPCR	Potentially
	Dead cells by cell viability measurement (see above)	Yes
	Apoptotic cells by flow cytometry	Yes
Process impurities		
	Residual FCS by BSA ELISA	No
	Residual antibiotic by LC-MS	No
	Residual trypsin by ELISA	No
	Residual beads by flow-imaging or optical microscopy	No
Potency		
	Secretion of cytokines by ELISA in cell culture assay with target cells	Potentially
	Transgene expression by flow cytometry	Potentially
	Target gene expression by real-time qPCR	Potentially
	Cytotoxicity assay	Potentially
	Change in phenotypic marker pattern by flow cytometry	Potentially
	In-vivo animal disease model (e.g., tumor burden; insulin production)	Potentially
Safety		
	Sterility by automated rapid microbial detection system	No
	Endotoxin by LAL	No
	Mycoplasma by NAT	No
	RCL by VSV-G qPCR	No
	Human and animal viral adventitious agents by e.g., NAT, PCR, Immunofluorescent antibody staining	No
General		
	Appearance by visual inspection	Potentially
	pH by potentiometry	Potentially
	Osmolality by osmometry	Potentially
	Visible foreign particulate matter by visual inspection	Potentially

[a] Abbreviations: FCS = fetal calf serum; HSA = human serum albumin; LAL = Limulus amebocyte lysate; NAT = nucleic acid amplification techniques; qPCR = quantitative PCR (polymerase chain reaction); RCL = replication competent lentiviral vector; STR = short tandem repeat; VSV-G = vesicular stomatitis virus G.

not a mainstream activity in formulation and stability studies. Reasons for this may be a lack of precision and difficulties in interpretation.

Identity and Purity Assays

Flow cytometry is also being used for measuring cell surface markers as indicators of API identity and purity (see Table 5). However, the results may not always be discriminative among CBMP formulation candidates for similar reasons as those mentioned for viability assays.[59] Moreover, for some CBMPs, such as mesenchymal stromal cell-derived products, defining API purity and (un)wanted cell populations is not straightforward. Therefore, the relationship between cell surface marker expression and product quality is not always readily established. Nevertheless, variations in their patterns during production, storage and upon administration have been observed. On the one hand, this suggests that cell surface marker assays are potentially useful in formulation screening. On the other hand, it leaves us with the question: what is the relevant information?

Potency Assays

The challenges described above also apply to potency assays. Potency is defined as the quantitative measure of biological activity (or activities) based on the product attribute(s) linked to the *in-vivo* mode of action (MoA) and clinical effect(s).[60] The MoA of a CBMP is often multifactorial, complex and not well understood, especially not in pre-clinical and early clinical phases. Therefore, complementary potency assays, measuring different aspects of the cell's hypothesized *in-vivo* function(s), are often used in formulation and stability studies. It is a common strategy to choose a combination of biochemical assays, biophysical assays, cell-culture based biological assays and, if available, animal-based biological assays (cf. Table 5).[61] Some tests are a more direct measure of potency (e.g., tests using an animal disease model) than others (e.g., an IFN-gamma ELISA). However, *in-vivo* models are generally less precise than *in-vitro* assays, are more expensive and more time consuming. Moreover, for many diseases, such as graft versus host disease and other immune disorders, establishing an appropriate animal model to investigate the CBMP's efficacy is challenging.[62] In such cases, data of various *in-vitro* potency tests (see Table 5 for examples) may be evaluated in conjunction with pharmacokinetic and pharmacodynamic study read-outs in post-administration patient materials. Such read-outs may include the measurement of T-cell subpopulations (e.g., T helper cells, cytotoxic T cells, memory T cells) in blood and bone marrow samples by flow cytometry. This approach may provide insight into relevant potency assays for product characterization, including formulation and stability testing.[8]

Analytical Method Development: Quo Vadis?

Evidently, there is a need for a better filled toolbox with sensitive, stability-indicating orthogonal and complementary analytical methods to assess CBMP quality and stability. Ideally, all of these should be fast, easy-to-perform, low-volume methods, in particular for non-off-the-shelf CBMPs where little material is present for formulation activities and for CBMPs with a short shelf-life.

Advanced characterization techniques, such as those based on genomics, transcriptomics, proteomics, secretomics and metabolomics, may shed light on the impact of different excipients and their concentrations on cell characteristics in general and CQAs in particular.[1,63] Examples of potentially useful techniques include cytometry by time-of-flight (CyTOF; for assessing purity and cellular impurities) and functional assays, such as secretome analysis.[64] Whole genome sequencing by next generation sequencing (NGS) (genomics) may be useful for genotypic stability assessment. Since mRNAs and miRNAs are involved in response to environmental stimuli, apoptosis, and metabolic activities,[5,11] alterations in their levels may provide insight into the impact of stress factors on cell product quality. These techniques could also be applied to compare CBMPs stored under fresh and frozen conditions. Protein profiles expressed in the cell or secreted into the environment, as measured by ELISA, LC-MS, NMR, 2D gel electrophoresis and immunoassays (proteomics and secretomics) as read out for API purity and potency (i.e., by investigating protein profile changes upon cell stimulation) could be established as standard techniques in formulation screening.[63] Environmental stress, including changes in pH, osmolality and temperature can induce changes in cell metabolism, which can be measured by LC-MS and LC-NMR (metabolomics).

Whether the above-mentioned 'omics' techniques are sufficiently sensitive and stability indicating still is an open question. Because of the limited availability of CBMPs, we may have to start with applying these tools in formulation and stability assessment of cell lines, such as Jurkat (T cell), mesenchymal stem cell and NK cell lines.[33,34] Altogether, the data generated may serve as a 'fingerprint' of CBMP quality and as such be employed to monitor product stability. Since all these approaches create a lot of information, artificial intelligence tools may be needed for data interpretation.

Remarkably, techniques that are commonly used to assess microparticulate impurities in classical biopharmaceuticals are not yet widely used for the characterization of CBMPs, i.e., products where cells (i.e., 'living microparticles') are the API. Nevertheless, particle counting and sizing techniques could be very useful for CBMP characterization and stability assessment. For instance, flow imaging microscopy (FIM) has been reported to be a sensitive tool for determining total cell concentration and cell viability as a function of storage time as well as process impurity assessment.[65,66] Therefore, it is worthwhile to perform further studies to explore the full potential of FIM and other image-based techniques for CBMP stability assessment.

Finally, connecting analytical quality data to non-clinical and clinical data is essential for a rational design of CBMP formulations and selecting storage conditions, as discussed earlier. There are still a lot of unknowns about the 'structure'-function relationship of these heterogeneous and complex CBMPs. This calls for an initiative such as the A-Mab and A-VAX projects where similar questions were addressed for other complex medicines, bringing together and analyzing the wealth of data industrial stakeholders collected over the years.[67,68] Assessing CQAs and providing design space information would give formulation scientists and regulators much needed guidance.

Conclusions and Recommendations

Many of the current CBMPs are modified cells of autologous origin. These are medicines tailored to the individual patient, for which small-scale manufacturing and dedicated QC strategies apply. With the expected advent and growing number of off-the-shelf products this situation will change. Larger-scale production batches will become the standard. Consequently, questions around formulation design will come to the forefront. Answering these questions will require extensive research on formulation development.

There is a need to simplify the present means of transport of sub-zero cooled CBMPs, with the introduction of CBMPs in dried form being the most prominent on the list. Major efforts should be made to come up with a dried CBMP that can be stored and shipped at refrigerator or ambient temperatures, to be reconstituted at the bedside and administered.

As long as we are stuck with frozen (cryopreserved) off-the-shelf CBMPs, it is highly desirable to develop simpler formulations without compromising CBMP stability. Additionally, the development of formulations allowing storage at −80 °C would alleviate issues with storage and logistics of products currently stored in liquid nitrogen tanks. Chemically defined formulation buffers, based on pharmacopeial grade excipients and devoid of biological compounds are high on the wish list. Ready-to-use proprietary formulation buffers may not be the first choice, as the quantitative composition and the manufacturing details are not known to the CBMP developer. Furthermore, there is a strong demand for replacement of DMSO by safer CPAs, which also may facilitate handling and improve the in-use stability of CBMPs post-thawing. Obviously, appropriate freezing and thawing procedures as well as storage, shipment and administration conditions must be established as part of formulation development. Finally, although the field has evolved from multiple, manual, conventional batch centrifugation wash cycles toward platforms that are functionally closed, automated and single-use,[1] closed integration with neighboring unit operations such as formulation and fill & finish steps remains a challenge. Nevertheless, the production field calls for initiatives to develop such integrated technologies.

For non-off-the-shelf CBMPs, liquid (fresh) formulations, stored in the refrigerator, or ideally at room temperature, are recommended. If this is not feasible, similar approaches as for off-the-shelf products apply. For fresh CBMPs, improved formulations that would allow shelf-life extension are very welcome. A prolongation of the shelf-life with only a few days or weeks would already facilitate logistics and possibly allow for full QC testing for batch release before administration.

While the need for improved formulations is high, developing such CBMP formulations is more easily said than done. An important limiting factor for non-frozen cells is the small amount of available cellular API. Although the optimal formulation probably depends on the cell type, we encourage the use of relevant test cell lines for formulation and stability studies to overcome the limited availability of verum CBMPs. Despite the limitations of this approach, these cell lines may be essential research and development tools to improve our fundamental insights into the impact of formulation, storage, transportation and handling on the quality and stability of different cell types. Similarly, these cell lines may prove indispensable for developing relevant analytical methods to assess cell quality and stability in a more accurate way compared to the current state of the art. Improved analytical tools are urgently needed to facilitate future CBMP formulation development exercises. The focus should be put on rapid, low-volume analytics, which is particularly important for permitting full release testing prior to administration of short shelf-life products.

As discussed above, currently several non-standard excipients are being used in CBMP formulations. If some of these turn out to be irreplaceable, then compendial guidance should be extended over the years to assist in ensuring the chemical quality of such excipients. Moreover, this could also lead to a re-appreciation of certain excipients that are at present commonly used, such a DMSO and materials from human or animal origin.

Formulation and fill & finishing steps should ideally be integrated in a closed, automated manufacturing process. This should include appropriate primary packaging materials. Whatever the final formulation composition will be, ultimately all excipients present in the CBMP formulation, their grade and quantities, the primary packaging materials and the storage conditions need to be justified in regulatory filings.

Defining relevant quality criteria is primarily a task for industry and regulatory bodies. So far, literature on the assessment of CQAs and design space for CBMPs is hardly available and/or seems to miss a solid base. The field would benefit from an initiative by major industrial players to bring together their experience and set the stage for establishing CQAs and design space for CBMPs, in analogy with the A-Mab and A-VAX projects. An initiative of regulatory bodies to harmonize the regulatory protocols for CBMP formulation design and acceptability would be applauded as well.

One may raise the question whether for non-off-the-shelf (autologous) CBMPs different quality criteria may apply than for off-the-shelf CBMPs. If so, what is acceptable for these products, especially in the case of production of hospital exempted (non-commercial) products? Do we accept wider release and stability acceptance criteria (design spaces) for products manufactured on demand at or close to the clinical site without extensive formulation studies?

Paradoxically, while CBMPs are categorized as ATMPs (A = advanced), CBMP formulation development is not yet in an advanced stage today. Nevertheless, the formulation determines to a large extent CBMP quality and stability, which encompasses clearly more than (cell) life or death. Moving the CBMP formulation field forward will require a substantial team effort, with involvement of experts from several disciplines, such as pharmaceutical scientists, analytical experts, product development specialists, cell biologists, cryobiologists, clinicians and regulators. This should eventually lead to the development of truly advanced formulations containing 'alive and kicking' cells that are potent, safe and stable as well as easy to handle in the clinic.

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