Recombinant pharmaceuticals from microbial cells: a 2015 update

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Abstract
Diabetes, growth or clotting disorders are among the spectrum of human diseases related to protein absence or malfunction. Since these pathologies cannot be yet regularly treated by gene therapy, the administration of functional proteins produced ex vivo is required. As both protein extraction from natural producers and chemical synthesis undergo inherent constraints that limit regular large-scale production, recombinant DNA technologies have rapidly become a choice for therapeutic protein production. The spectrum of organisms exploited as recombinant cell factories has expanded from the early predominating *Escherichia coli* to alternative bacteria, yeasts, insect cells and especially mammalian cells, which benefit from metabolic and protein processing pathways similar to those in human cells. Up to date, around 650 protein drugs have been worldwide approved, among which about 400 are obtained by recombinant technologies. Other 1300 recombinant pharmaceuticals are under development, with a clear tendency towards engineered versions with improved performance and new functionalities regarding the conventional, plain protein species. This trend is exemplified by the examination of the contemporary protein-based drugs developed for cancer treatment.

Keywords: Recombinant proteins, Protein drugs, Recombinant DNA, Fusion proteins, Biopharmaceuticals

Background
Human cells produce thousands of proteins that integrated into an extremely complex physiologic network perform precise actions as catalysts, signalling agents or structural components. Then, dysfunction of proteins with abnormal amino acid sequences or the absence of a given protein often results in the development of severe pathologies such as diabetes [1], dwarfism [2], cystic fibrosis [3], thalassaemia [4] or impaired blood clotting [5], among many others [6, 7]. In the absence of standardized gene therapy treatments that would genetically reconstitute functional protein production within the patient, protein deficiencies must be treated by the punctual or repeated clinical administration of the missing protein, so as to reach ordinary functional concentrations. These therapeutic proteins are produced ex vivo mostly in biological systems [8], which must guarantee not only full protein functionalities but also a cost-effective industrial fabrication and the absence of hazardous contaminants. Protein drugs have to necessarily conform to quality constrains stricter than those expected in the production of enzymes for chemical industries, which consequently defines the choice of recombinant hosts, protocols and production strategies. Nowadays, there are over 400 marketed recombinant products (peptides and proteins) and other 1300 are undergoing clinical trials (figures updated on May 2015 [9]).

In this context of expanding protein drug markets, there is a generic consensus about the need to enable drugs for cell- or tissue-targeted delivery to reduce doses, production costs and side effects. While increasing protein stability in vivo can be reached by discrete modifications in the amino acid sequence, generating fusions between therapeutic proteins and specific peptide ligands or antibodies that interact with particular cell receptors might allow acquiring specificity in the delivery process.

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In this regard and also pushed by the convenience to combine diagnosis and therapy in theranostic agents [10, 11], contemporary research on protein pharmaceuticals tends towards engineered versions functionally more sophisticated than plain natural polypeptides.

**Review**

**Cell factories**

Since early recombinant DNA times, ever-increasing understanding of cell physiology and stress, and of factors involved in heterologous gene expression and protein production empowered the use of different living factories, namely prokaryotic and eukaryotic cells, plants or animals [12, 13]. By using these systems, recombinant production solves source availability problems, is considered a bio-safe and green process and confers the ability to modify amino acid sequences and therefore protein function, to better adjust the product to a desired function [14]. There is a wide and growing spectrum of expression systems that are becoming available for the production of recombinant proteins [15, 16]. *Escherichia coli* was the prevalent platform when the biopharmaceutical sector emerged in the 1980s, and it was followed by the implementation of the yeast *Saccharomyces cerevisiae*. Both systems and the associated genetic methodologies exhibit an unusually high versatility, making them adaptable to different production demands [17]. Despite the exploration of insect cells as initially successful system especially for vaccine-oriented proteins, mammalian cell lines (most notably CHO cells) are nowadays the prevailing animal-derived cell system due to their suitability to produce conveniently glycosylated proteins [18, 19] (Fig. 1). The ability to carry out post translational modifications contrasts with complex nutritional requirements, slow growth and fragility, and relatively high production timing and costs. Thus, among many conventional and emerging cell-based systems for protein production, bacteria, yeast and mammalian cell lines are the most common in biopharma, and both prokaryotic and eukaryotic systems are constantly evolving and competing to improve their properties and intensify as platforms of choice for protein drug production [14]. While bacteria has lost its early leading role in the field [19], about 30 % of marketed biopharmaceuticals are still produced in this system [20], as supported by the unusual physiological and genetic manipulability of prokaryotic cells [21].

In fact, the main purpose in the development of new protein production platforms is to enhance drug functionality through reaching successful protein folding and post-translational modifications, while keeping the low complexity and high flexibility associated to prokaryotic cell culture. In this context, Gram-positive bacteria such as *Bacillus megaterium* [22] and *Lactococcus lactis* [23] allow efficient protein secretion in absence of endotoxic cell wall components, while filamentous fungi (such as *Trichoderma reesei*, [24]), moss (*Physcomitrella patens*, [25, 26]) and protozoa (*Leishmania tarentolae*, [27–29]) promote glycosylation patterns similar to those in mammalian proteins but being still cultured through methods simpler than those required by mammalian cells. Extensive descriptions of emerging (bacterial and non-bacterial) platforms specifically addressed to the production of high quality protein drugs can be found elsewhere [15, 16, 21]. The recent development of an endotoxin-free strain of *E. coli* [30] and its application to the fabrication of proteins and protein materials [30–32] paves the road for a cost-efficient and versatile production of proteins intended for biomedical uses by skipping endotoxin removal steps, thus gaining in biosafety and reducing production costs [33]. Hopefully, all these new systems would soon offer improved products in still simple and fully controlled biofabrication approaches.

**Trends in protein biopharmaceuticals**

Nearly 400 recombinant protein-based products have been successfully produced and are approved as biopharmaceuticals [9], a term that refers to therapeutic products generated by technologies that involve living organisms [34]. Other 1300 protein candidates are under development, of which around 50 % are in pre-clinical studies and other 33 % in clinical trials [9] (Fig. 2). In this context, an increase in the number of approvals in next years is predictable. Developed by Eli Lilly & Co in the 70’s, Humulin, a recombinant human insulin fabricated in the bacterium *E. coli* [35], was the first approved biopharmaceutical (by the FDA) in 1982 [36, 37]. Other natural proteins such as hormones, cytokines and antibodies (Orthoclone OKT3) were among the single nine products approved in 1980s (Table 1). Nowadays, the therapeutic areas that have benefited more from recombinant biopharmaceuticals are metabolic disorders (e.g. diabetes
type 1, type 2, obesity or hypoglycaemia), haematological disorders (e.g. renal anaemia, haemophilia A, bleeding or clotting disorders) and oncology (e.g. melanoma, breast or colorectal cancer), with 24, 18 and 15 % of the approvals respectively (Fig. 3). In this regard, oncology is a clearly expanding market. In the period 2010–2014, 9 out of 54 approved biopharmaceuticals were antitumoral drugs, cancer representing the most common indication within this period. Digging into the molecular bases of biopharmaceuticals, there is a clear trend towards antibody-based products. Over the same period (2010–2014), 17 of the 54 protein drugs approved were monoclonal antibodies (31.5 %), compared with 11 % over 1980–1989 [22]. Furthermore, among the top ten selling protein biopharmaceuticals globally in 2014 (Table 2), six are antibodies or antibody-derived proteins (Humira, Remicade, Rituxan, Enbrel, Avastin, Herceptin; http://qz.com/349929/best-selling-drugs-in-the-world/).

Formerly, biopharmaceuticals were recombinant versions of natural proteins, with the same amino acid sequence as the respective native versions (with only minor modifications, often resulting from the cloning strategy). Since 1990s, a meaningful proportion of the approvals are based on highly modified forms of recombinant proteins. This novel alternative, based on protein or domain fusion and on truncated versions, offers a wide spectrum of possible combinations to obtain novel biopharmaceuticals with different joined activities that are not found together in nature.

### Protein drugs for cancer treatment

Oncology is one of the therapeutic indications that dominate the biopharmaceutical market, as cancer is a major cause of morbidity and mortality worldwide. Surgery and radiotherapy are effective in curing cancer at early disease stages; however, they cannot eradicate metastatic disease. The presence of micrometastases or clinically evident metastases at diagnosis requires their use in combination with genotoxic chemotherapy to increase cure rates [38]. Nevertheless, the success of chemotherapy has been hampered because of its lack of selectivity and

| Table 1 Recombinant biopharmaceuticals approved in the 1980s |
|--------------------------------------------------------------|
| **Product** | **Cell factory** | **Therapeutic indication** | **Year** |
|---|---|---|---|
| Humulin | *E. coli* | Diabetes | 1982 |
| Protropin | *E. coli* | hGH deficiency | 1985 |
| Roferon A | *E. coli* | Hairy cell leukaemia | 1986 |
| IntronA | *E. coli* | Cancer, genital warts and hepatitis | 1986 |
| Recombivax | *S. cerevisiae* | Hepatitis B | 1986 |
| Orthoclone OKT3 | Hybridoma cell line | Reversal of acute kidney and transplant rejection | 1986 |
| Humatrope | *E. coli* | hGH deficiency | 1987 |
| Activase | CHO | Acute myocardial infarction | 1987 |
| Epogen | CHO | Anaemia | 1989 |
specificity, so that the toxicity to normal tissues limits the dose that could be administered to patients. The development of biopharmaceuticals capable of inhibiting specific molecular targets driving cancer (for instance, monoclonal antibodies anti-Her2—Trastuzumab- or anti-VEGF—Bevacizumab-) goes in this direction [39].

Among marketed protein biopharmaceuticals, almost 24% (94 products) are used in antitumoral therapies. Most of these products are used for supportive purposes intended to minimize the side effects of chemotherapy, usually neutropenia or anaemia (some representative examples are shown in Table 3). Nineteen out of those 94 products are true antitumoral drugs, 69% of which are produced in \( E. coli \) (Fig. 4) and are based on engineered amino acidic sequences, protein fusions and single protein domains (Table 4).

Clearly, modified protein versions are the most abundant in cancer therapies over natural polypeptides. As relevant examples, Ziv aflibercept is a recombinant fusion protein produced in CHO cells used against colorectal cancer. It consists of portions of each Vascular Endothelial Growth Factor Receptors (VEGFR1 and VEGFR2) fused

### Table 2. Top ten selling protein biopharmaceuticals in 2014

| Drug* | Active ingredient | Molecule | Sales in billions | Origin |
|-------|-------------------|----------|-----------------|--------|
| Humira | Adalimumab | Recombinant human monoclonal antibody | 12.54 | CHO |
| Sovaldi | Sofosbuvir | Nucleotide analogue polymerase (NS5B) inhibitor | 10.28 | Chemical |
| Remicade | Infliximab | Recombinant chimeric, humanized tumor necrosis factor alpha (TNF) monoclonal antibody | 9.24 | Hybridoma cell line |
| Rituxan | Rituximab | Recombinant humanized monoclonal antibody | 8.68 | CHO |
| Enbrel | Etanercept | Recombinant soluble dimeric fusion protein | 8.54 | CHO |
| Lantus | Insulin glargine | Insulin receptor agonist | 7.28 | \( E. coli \) |
| Avastin | Bevacizumab | Recombinant humanized antibody | 6.96 | CHO |
| Herceptin | Trastuzumab | Recombinant humanized monoclonal antibody | 6.79 | CHO |
| Advair | Fluticasone propionate and salmeterol xinafoate | Glucocorticoid receptor agonist and \( \beta \)-2 adrenergic receptor agonist | 6.43 | Chemical |
| Crestor | Rosuvastatin calcium | Antihyperlipidemic agent | 5.87 | Synthetic |

* Data according to [www.medtrack.com](http://www.medtrack.com), November 2015

### Table 3. Representative examples of supportive protein drugs in cancer

| Drug name | Cell factory | Biological role | Mechanism of action | Indications |
|-----------|--------------|-----------------|---------------------|-------------|
| Filgrastim (Scimax) | \( E. coli \) | Cytokine | Stimulates hematopoiesis | Bone marrow transplantation and cancer chemotherapy induced neutropenia |
| Pegfilgrastim (Neupeg) | \( E. coli \) | Cytokine | Stimulates differentiation, proliferation and activation of the neutrophilic granulocytes | Cancer chemotherapy induced neutropenia |
| Darbepoetin alfa (Aranesp) | CHO cells | Hormone | Stimulates processes of erythropoiesis or red blood cell production | Anemia associated with chronic renal failure, cancer chemotherapy or heart failure. Myelodysplastic syndrome |
| Lenograstim (CERBIOS) | CHO cells | Cytokine | Stimulates differentiation, proliferation and activation of neutrophilic granulocytes | Neutropenia associated with cytotoxic therapy or bone marrow transplantation |
| Epoetin alfa (Binocrit) | CHO cells | Hormone | Stimulates production of oxygen carrying red blood cells from the bone marrow | Anemia associated with chronic renal failure and cancer chemotherapy induced anemia |

![Fig. 4](image-url) Cell factories used for the production of recombinant biopharmaceuticals against cancer (expressed in percentages)
to the constant fraction (Fc) of a human IgG1 immunoglobulin (Fig. 5). This construct acts as a decoy by binding to VEGF-A, VEGF-B and placental growth factor (PIGF), which activate VEGFR. This trap hinders the interaction between the growth factors and the receptors, inhibiting the VEGF pathway which is involved in the angiogenic process [40]. Denileukin diftitox is a recombinant protein composed of two diphtheria toxin fragments (A and B) and a human interleukin-2 (Fig. 5). Diphtheria toxin is a potent exotoxin secreted by *Corynebacterium diphtheriae*. Due to its peculiar structure, the whole complex, produced in *E. coli*, is capable of delivering a cytotoxic agent directly to a specific target. There are two main active blocks whose function is firstly to selectively deliver the biopharmaceutical (IL-2) and secondly cause cytotoxicity (toxin A and B) [41]. The fusion protein binds to the IL-2 receptor, which is expressed in cancerous cells (cutaneous T-cell lymphoma). Once the toxin moiety is internalized, the catalytic domain promotes cell death through protein synthesis inhibition [42].
As targeted drug delivery for cancer is a most recent and expanding area of research, other non-recombinant, protein-based biopharmaceuticals are also heavily represented. Those mainly include antibody-drug conjugates (ADCs) such as Brentuximab vedotin, Trastuzumab emtansine, or nanoparticle-drug conjugates such as nab-paclitaxel [39, 43]. In these cases, the protein counterpart acts as a targeted vehicle for conventional chemical drugs. Again, this approach pursues the selective drug delivery to specific target cells, aimed to increase antitumoral activity while reducing toxicity on normal cells and the associated side effects.

Products against cancer that provided the highest revenues in 2013 are represented in Fig. 6. Sixty percent of those products are recombinant proteins, supporting the idea that recombinant protein production is still a rising and promising platform, offering room for important advances in the biopharmaceutical sector.

Conclusions
In summary, the market and potential for recombinant drugs is expanding by taking advantage of a steady growing spectrum of protein production platforms. Despite the strength of mammalian cell lines as factories, microbial cells and specially E. coli are still potent protein factories essentially supported by their versatility and cost-effective cultivation. Recombinant drugs are moving from plain recombinant versions of natural products to more sophisticated protein constructs resulting from a rational design process. Combining protein domains to gain new functionalities is being exploited in drug discovery by exploiting the structural and functional versatility that merge in proteins as extremely versatile macromolecules.

Abbreviations
AIDS: acquired immune deficiency syndrome; ADCs: antibody-drug conjugates; CHO: Chinese hamster ovary; CML: chronic myelogenous leukemia; Fc: constant fraction; FDA: food and drug administration; hGH: human growth hormone; IL: interleukin; PlGF: placental growth factor; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor.

Authors’ contributions
LSG performed most of the bibliographic search and prepared part of the text, tables and figures, under the supervision of NFM and EV. LM and RM contributed with additional information and revised the manuscript. AV coordinated the whole revision, prepared part of the text and figures and the final manuscript version. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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