Advanced materials from microbial fermentation

The case of glycolipids and nanocellulose

Résumé **Matériaux avancés issus de la synthèse microbienne : exemples des glycolipides et de la nanocellulose**
La chimie verte est une discipline relativement récente régie par douze principes fondateurs, incluant notamment l'économie d'atomes, la prévention de la pollution *via* des méthodes de synthèse chimique respectueuses de l'environnement, comme par exemple celles privilégiant le milieu aqueux aux solvants organiques, mais aussi le développement de produits chimiques et matériaux issus de la biomasse végétale. Dans ce contexte, la synthèse microbienne est un outil de choix pour supplanter dans certains cas les approches classiques basées sur la chimie organique de synthèse. La synthèse microbienne de composés sucrés polymères ou lipidiques progresse et ne se limite plus à la communauté des chercheurs en microbiologie, historiquement intéressés au développement des produits de fermentation de microorganismes. Cet article présente la production, la diversification et l'étude des propriétés de composés sucrés en se focalisant sur la nanocellulose bactérienne pour les polymères glycosylés, et les biotensioactifs pour les systèmes lipidiques glycosylés. Le choix de ces deux systèmes est justifié par le fort développement des matériaux à base de nanocellulose et le besoin de remplacer en partie les tensioactifs « classiques », sources non négligeables d'émissions de CO_2 au niveau mondial.
Mots-clés **Chimie durable, matériaux, fermentation microbienne, cellulose bactérienne, nanocellulose, glycolipides, biotensioactifs.**

Abstract Green chemistry is a recent discipline ruled by twelve founding principles, which include, among others, atom economy, the prevention of pollution *via* environmentally friendly chemical synthesis methods, such as, for example, the choice of an aqueous medium over organic solvents, but also the development of chemicals and materials derived from plant biomass. In this context, microbial synthesis is a tool to supplant, in some notable cases, syntheses by a standard organic chemistry approach. More recently, attention has begun to be given to the microbial synthesis of polymeric sugars, such as dextran or cellulose, or lipids, such as amphiphilic glycolipids. Although the microbial production of glycosylated compounds can be traced back by several decades, the development of green chemistry is encouraging teams of multidisciplinary researchers to focus on production, diversification, and applications of this class of compounds, thus going beyond the community of researchers in microbiology, historically interested in the development of fermentation products from microorganisms. This article develops the above-mentioned theme by focusing on nanocellulose, representing an important glycosylated polymer, and on biosurfactants, in regards of the glycosylated lipids. The choice of these two systems is justified by the strong development of nanocellulose-based materials but also by the need to replace in part the "conventional" surfactants, a significant source of CO_2 emissions worldwide. The main classes of molecules, the classical methods of synthesis, their properties and some examples of notorious applications are presented.
Keywords **Sustainable chemistry, materials, microbial fermentation, bacterial cellulose, nanocellulose, glycolipids, biosurfactants.**

Strategic raw materials: nanocellulose and glycolipids

Nanocellulose as a term originated only in the first decade of the 2000s [1] but many of the current nanocellulose species had been in existence at a much earlier date [2]. In essence, nanocellulose is generally categorized under three discrete groups: cellulose nanocrystals (CNCs), cellulose nanofibers (CNFs), and bacterial cellulose (BC) [1].

BC is the odd one out of the nanocellulose family. It consists of cellulose microfibrils synthesized by certain bacteria to an extracellular matrix [3]. BC microfibrils are in a completely isotropic arrangement, lacking the tight hierarchical morphology of plant cellulose confined in the cell wall (*figure 1a*). Therefore, one can say that BC is directly synthesized as a nanocellulose species without the need for specific isolation from a growth matrix. BC is also the only chemically pure form of nanocellulose as the other species are always embedded in a matrix of hemicellulose and – with land plants – lignin which are next to impossible to remove completely during nanocellulose preparation [4].

Only certain types of bacteria are able to synthesize BC from glucose. The most popular species for actual BC production is called *Acetobacter xylinum*, or *Gluconacetobacter xylinus* in modern nomenclature. The BC synthesis was first reported in 1886 [5], although the product was only much later identified as cellulose [6]. Culturing with *G. xylinus* is an aerobic process where BC grows at air/water surface in order to utilize the oxygen from air [3, 7]. The most used monosaccharide sources for BC production are glucose and sucrose. Glucose is converted *via* several steps into uridine diphosphoglucose (UDP-glucose) which is then polymerized into cellulose, i.e. a homopolymer consisting only of anhydroglucose units (*figure 1d*) [3, 7]. BC producing microbes are also able to utilize other monosaccharides to produce UDP-glucose and further to cellulose. A case in point is fructose – the other monosaccharide in the sucrose dimer –, but several other monosaccharides have been trialed as a cellulose source

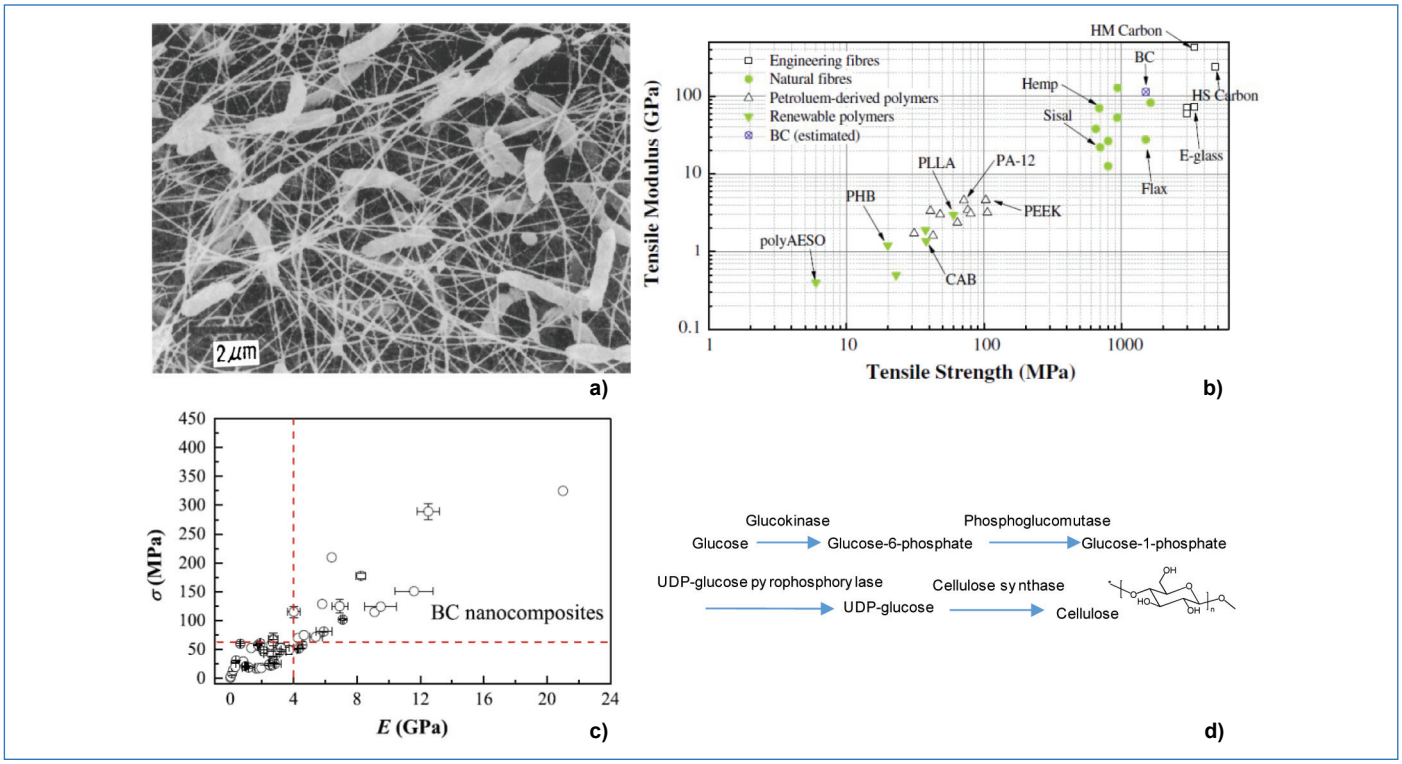

Figure 1 - a) Scanning electron micrograph of bacterial cellulose gel. The gel still contains the bacteria which are generally removed after culturing by a simple washing step. Reproduced from [3], © Springer 2000. b) Average tensile properties of BC in comparison to some commonly used synthetic and renewable fibers. c) Tensile strength versus tensile modulus of selected BC composites. The dashed lines denote tensile properties of neat PLA. Reproduced from [8], © Elsevier 2014. d) Simplified pathway from glucose to bacterial cellulose.

as well [3]. Commonly, the method of growing BC in static conditions at the air/water surface is relatively slow but nevertheless suitable for laboratory work.

The peculiar feature of BC is that it forms a hydrogel pellicle after the synthesis. The pellicle consists of isotropic BC microfibrils. Additionally, BC is exceptionally effortless to purify from other components that cellulose; a mild alkali washing is sufficient to remove the bacteria and additional sugars. In other words, BC is a nanocellulose hydrogel in a very pure form, which is something that many nanocellulose procedures are attempting to achieve from plant materials with much effort. It is, therefore, quite an obvious development that BC has been subjected to similar investigations as the rest of the nanocellulose family, composite materials being among the most popular targets for applications. Furthermore, biomedical applications, particularly wound dressing materials, have been fashionable with BC because of its chemical purity and non-toxicity.

Biomedical applications are another strong research trend with BC-containing materials. In particular, wound dressing has received a lot of attention with even commercial or pre-commercial products in the line [9]. Here, the high mechanical strength of BC, its hygroscopicity, non-toxicity, antimicrobial nature, and ability to geometrically conform in various shapes are seen as the principal assets. The similarity of BC nanofibers with collagen fibers of the skin improves the biocompatibility. Other biomedical applications of BC include tissue-engineering [10] and drug delivery [11].

Biosurfactants: market, constraints and actors

Surfactants are performance molecules that intervene in nearly every product and aspect of human daily life and find applications in a very broad array of markets and applications: chemical, pharmaceutical, cosmetic and personal care, packaging, carpets and textiles, detergents, paper, adhesives, (3D printing) inks, mining and leaching, healthcare, polymer, food and feed, paints, surface and industrial coating, etc. The global turnover of surfactants was worth US$ 30.64 billion in 2016 and is expected to reach US$ 39.86 billion by 2021, registering a CAGR (compound annual growth rate) of 5.4% between 2016 and 2021 [12], which shows that they are bulk products with a significant economic and environmental impact.

Despite the efforts to move towards a more environmental friendly economy, only 3% of the surfactant market share consists out of molecules which are 100% bio-based, and even a smaller part (< 0,5%) of entirely biologically produced bio-based surfactants, such as microbial, plant based or enzymatically produced biosurfactants. There are myriad pathways to process crude oil or gas towards the hydrophobic building blocks of synthetic surfactants, e.g. Fischer-Tropsch synthesis, oxo process, olefin oligomerization or Friedel-Crafts alkylation. The constant consumer and hence market demand for sustainable, bio-based and green solutions, has resulted in a substantial increase of the development and use of partly bio-based and even wholly bio-based surfactants (WBS) (100% derived from renewable (non-fossil) biomass in such applications [13]. *Figure 2a* displays the distribution of bio-, partially and non bio-based surfactants in the European market.

According to ISO 16128 norm, WBS represent about 3% of the European surfactant market and have the best image. Subsequently, there is a strong drive of the industry to apply this type of surfactants in their products, of course without giving in performance or cost. Most WBS are based on sugars coupled to fatty acids and/or alcohols, i.e. methyl ester sulphonates, alkylpolyglucosides, sorbitan esters, anionic APG derivatives, sucrose esters, methyl glucoside esters, fatty acid N-methylglucamides, alkylpolypentosides, etc.

However, some limitations/drawbacks are associated with the current WBS portfolio:
- the largest part of the wholly bio-based surfactants on the market is produced through chemical processes, which negatively impacts their environmental profile;
- their functionality/variety is limited;
- they do not offer an easy possibility for further derivatization/functionalization;
- they show good performance but do not boost performance by e.g. combining properties justifying their higher price;
- they can only be produced from first generation substrates.

Several new technologies are currently being developed, that can alleviate these issues. Related to the first hurdle, full biological production processes are associated with an even better environmental profile and additionally offer better marketing opportunities to the companies, which is a big driver.

Biosurfactants: a difficult adoption

The current state of the art for biologically produced surfactants (estimated by the authors to currently only account to a few thousand tonnes per annum, thus representing only a very small amount of the surfactant market share) can be defined as:
- plant derived molecules like e.g. saponins or cardolite commercialized by e.g. Foodchem, Dr. H. Schmittmann GmbH and Cardolite respectively;
- enzymatically produced molecules like e.g. enzymatic sugar esters, which are not on the market yet, but a lot of research is conducted in this field;
- microbially produced molecules like sophorolipids (SLs) launched on the business to business (B2B) market by Soliance, Evonik, planned by Croda, and applied by Ecover, Saraya, Henkel, Soliance, etc.; rhamnolipids (RLs) commercialized by Logos, Jeneil, Urumqi Unite Bio-Technology, Biotensidon, AGAE and Rhamnolipid Inc.; mannosylerythritol lipids (MELs) commercialized by Toyobo, Kanebo, Damy Chemical, Biotopia Co. and investigated by many more; lipopeptides (LPs) commercialized by Lipofabrik, Kanebo and Kaneka.

Microbial surfactants already offer a solution to the first drawback mentioned above: they can be produced from second generation (2G) substrates and waste streams like molasses, animal fats, dairy industry whey and other waste or side streams [14-15].

Three main reasons can be defined to explain low commercialization levels of biosurfactants. ***Cost considerations*** are to date the prime hindrance to market penetration of (microbial) biosurfactants. The maturity of the petrochemical industry makes purely cost-based competition unrealistic for most biosurfactants. Moreover, even the availability of biosurfactants with equivalent performance and at the same cost would not per se be sufficient to drive acceptance and utilization by consumers and brand owners. A better performance at an acceptable premium price would increase their marketability for mass consumption products as consumers nowadays demand environmentally friendly and benign products/processes, without wanting to give away functionality/performance.

A second hurdle is the ***lack of diversity***. Formulation experts are like artists, demanding a diversified "palette" of molecules to shake and stir to get to a desired endpoint. Whereas the chemical industry has been very successful in developing an extraordinary wide variety of surfactant structures for a multitude of applications/functions, the bio-based counterparts have not been able to offer the same diversity. To give an example, the chemically produced biosurfactants APGs (alkyl polyglucosides) mainly consist of mono-glucosylated (degree of polymerisation DP = 1) molecules and a small amount of di-glucosylated (DP = 2) molecules. Consequently, the structural diversity of these biosurfactants is rather low with an average DP ranging between 1.2 and 1.6. Although APGs are one of the most prominent biosurfactants on the market, their narrow product spectrum, and little opportunity for their derivatization/functionalization, limit further growth.

A third reason is the fact that (microbial) biosurfactants typically are defined by the occurrence of very similar congeners, i.e. ***production of mixtures***. For the microbial biosurfactants sophorolipids, these are mainly divided into a mixture of lactonic and acidic sophorolipids (*figure 2c*), although additional variations within the congeners (hydrophobic tail length, saturation degree, etc.) also occur. Easily over thirty congeners can as such be identified in "sophorolipids", which are thus not defined by one single molecule. This in product variation is one of the reasons of the very varying reports in the literature on their properties. The latter is simply unacceptable from an industrial point of view. Combined with the reasons mentioned above, this is one of the main reasons why microbial biosurfactants currently only represent a marginal part of the surfactant market.

A number of solutions can be defined for the mentioned bottlenecks. Variation can be increased by applying chemical and/or enzymatic derivatization possibly in combination with an expansion of the molecular variety through genetic engineering. The latter can also increase productivity (decrease of price) and uniformity (decrease of mixtures). The efforts spread over the past fifteen years [16], especially at the University of Ghent (Belgium) [17-19], have resulted in the generation of a proprietary platform technology for the production of new types of glycolipid molecules (*figure 2b*). This has been accomplished by the development of molecular techniques and constant expansion of the molecular toolbox for this non-conventional yeast and through the development and use of several -omic strategies. Although these engineering efforts have thus been quite successful, none of the above-mentioned efforts have yet resulted in the commercialization of a new-to-nature compound. This is due to a few reasons:
- new molecules translate/correspond with new processes, different properties and thus different applications;
- the non-optimized production processes and strains logically correspond with higher production costs;
- the absence of the molecules on the market works inhibitory for the economy of scale to kick in nor is regulatory approval granted (or data available for initiation of a regulatory dossier) although the related wild type SLs are;
- the lack of knowledge on the properties of these new molecules.

These hurdles are thus the next steps to take to valorize this technology and this is done by applying an integrative process design approach combining strain engineering with process development and optimization.

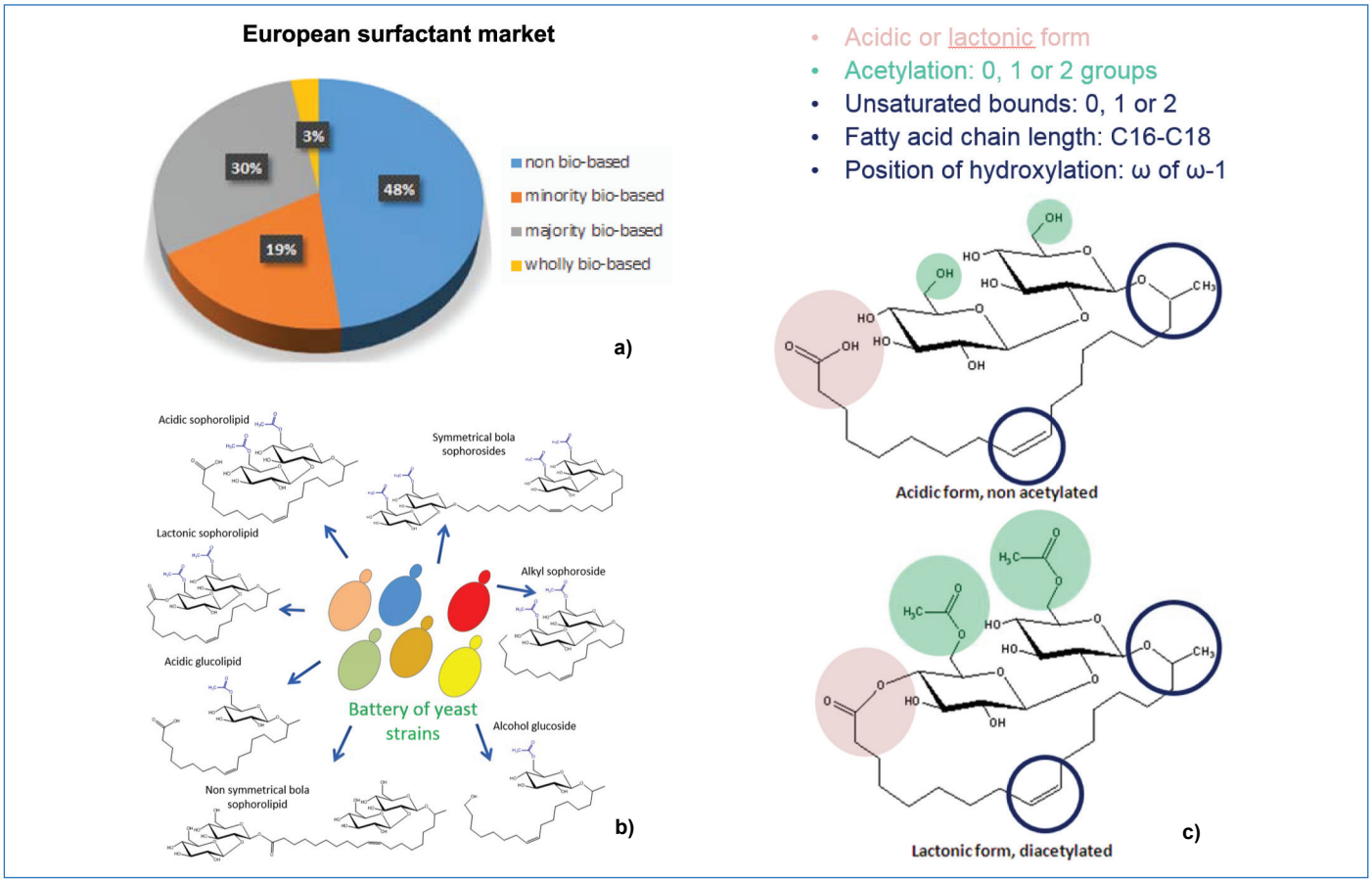

Figure 2 - a) Use of bio-based and non bio-based surfactants in the European Union, Norway, Switzerland and Iceland (2015) [13]. b) Glycolipid portfolio developed by InBio.be based upon the yeast *Starmerella bombicola*. c) Representation of variation in wild type sophorolipids.

Other perspectives are emerging

The discussion above mainly concerns the surface-active properties, by far the most important from an industrial development point of view, of microbial glycolipid biosurfactants. However, a series of new perspectives have been opened by research groups working mainly in Japan, India, France and USA. The biocompatibility of most glycolipids and the presence of the free carboxylic acid group made them interesting candidates for the water stabilization of metal and metal oxide nanoparticles. The first work in this field was proposed by the group of Prasad at the NCL in Pune, India. Cobalt [20], silver [21], gold [22] but also iron oxide [23] nanoparticles (*figure 3a*) have been used as systems to be coated with the acidic form of sophorolipids. In all cases, the resulting sophorolipid-coated system is highly dispersible in water and it was shown that, in the case of metal systems, sophorolipids can also act as a reducing agent, thus excluding the use of strong, classical, reducing agents like $NaBH_4$. Cytotoxicity and genotoxicity tests performed on the gold and silver nanoparticles systems have shown no specific biological activity below 100 µg/mL [22], thus making these systems interesting candidates for biomedical applications. Antimicrobial activity of glycolipid biosurfactants in solution is a field of research since a long time. However, more recent works have shown their interest as surface antimicrobial and/or antiadhesive coatings. Dispersion of rhamnolipids on both hydrophobic (octadecyltrichlorosilane-modified glass) and hydrophilic (hydroxyl-rich glass) surfaces was shown to have an interesting antiadhesive effect on Gram-negative *E. coli*, *P. putida* and *P. aeruginosa* and on Gram-positive *B. subtilis* [24] (*figure 3d*). On the contrary, if the glycolipid (sophorolipid) is chemically grafted on the substrate *via* its pending COOH group (amidation reaction with a surface aminothiol primer deposited on gold), biocidal properties against both Gram-positive (*L. ivanovii*, *E. faecalis*, *S. epidermidis*, *S. pyogenes*) and Gram-negative (*E. coli*, *P. aeruginosa*, *S. typhymurium*) bacteria are observed instead [25-26].

Surfactants are known to spontaneously self-assemble in water, and glycolipid biosurfactants do not derogate this rule. Self-assembly properties have been reported for mannosylerythritol lipids, rhamnolipids, sophorolipids, glucolipids, cellobioselipids, just to cite the most important ones [30-35]. If the knowledge in this field is more or less advanced according to the effort that a specific research group has dedicated to a given family of molecules, one can summarize, without being exhaustive, the following morphologies: micelles [30, 35-37], vesicles [30, 33-34], fibers [33-34], lamellar [30, 33], sponge [30] phases (*figure 3b-c*). The nature of the molecule and concentration are the most obvious parameters that influence the self-assembled morphology, but pH [32, 36, 38] and temperature [30, 32] have also been shown to have a strong influence on the self-assembly properties. This impressive load of work is still ongoing because the relationship between the structure of a given microbial glycolipid and the corresponding self-assembled phase is not obvious and cannot be based on the classical prediction based on packing parameter considerations, as recently shown for acidic oleic acid sophorolipids, which can form micelles or giant ribbons as a function of the purity but also, for a 100% pure compound, as a function of the preparation method [39]. One must here observe that microbial glycolipid systems are in fact never pure and in fact very hard to fully purify, because it is well known that

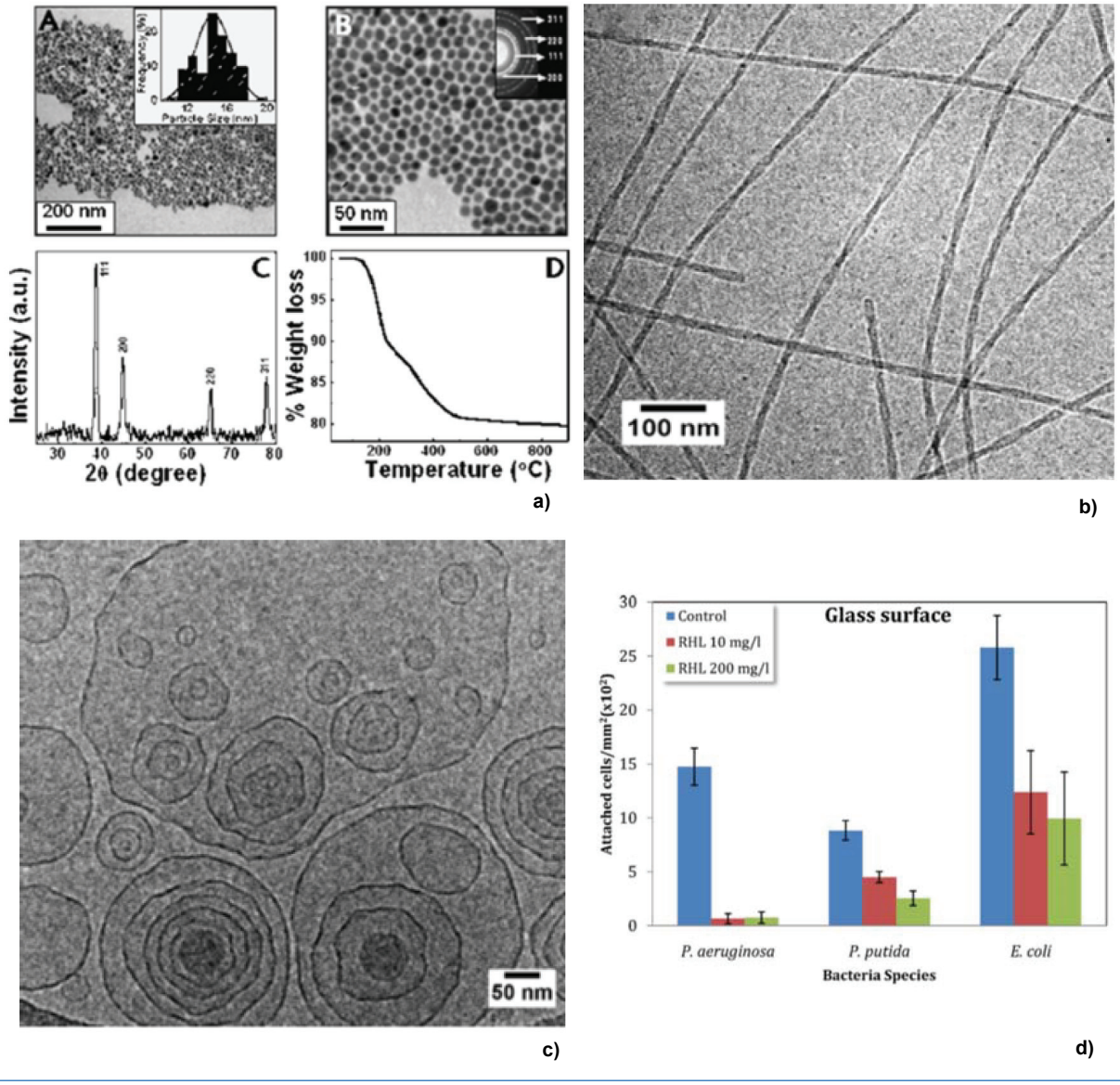

Figure 3 - a) Sophorolipid-capped silver nanoparticles (morphology, size, structure and organic content are respectively presented in panels A-B, C and D). Reproduced from [27]. © Royal Society of Chemistry 2008. b) Self-assembled nanoscale twisted ribbons obtained from stearic acid sophorolipids. Reproduced from [28]. © Wiley-VCH 2015. c) Glucosomes obtained from branched C22 sophorolipids. Reproduced from [29]. © Wiley-VCH 2017. d) Antiadhesive effects of rhamnolipids-coated glass substrate. Reproduced from [24]. © Wiley-VCH 2013.

microbial synthesis provide a system with a wide range of structurally-similar congeners where one or two are majoritarian.

Gene transfection, which consists in the delivery of genetic material across the cell membrane, is one of the applications in which control of self-assembly, and colloidal properties in general, is very important. In this field, mannosylerythritol lipids (MEL) have been employed as adjuvants in gene transfection of plasmid DNA using cationic liposomes as classical carriers and DNA binders [30]. It was shown that the presence of MEL increases the efficiency of gene transfection in NIH3, COS-7 and HeLa cells up to 50 to 70 times. The role of MEL seems to be the acceleration of membrane fusion between the cationic liposome and cell membrane, so that transfection efficiency is increased. More recently, quaternary ammonium-modified sophorolipids have been incorporated into negatively-charged DOPE liposomes and used as direct binders of negatively-charged plasmid DNA, to be transfected into A549, 16HBE and SKMEL28 cell lines [40]. Authors have found that two specific, long-chain, quaternary ammonium derivatives were highly efficient and with low cytotoxicity. The main difference between this work and the approach using MEL is the positively-charged sophorolipid obtained by chemical modification and its direct binding to plasmid DNA, thus giving it a direct role in terms of vectorization.

The present contribution shows two families of carbohydrate-rich compounds of microbial origin, one polymeric and the other molecular. Bacterial cellulose is an interesting polysaccharide challenging the use of plant cellulose for the easier approach in the purification and good water-dispersion properties. On the other side, microbial glycolipids are versatile compounds both in terms of lipid and carbohydrate structure that can self-assemble into a wide range of morphologies (micelles, vesicles, fibers…) and be used as antimicrobial and surface stabilizing agents, among others. Both families of compounds are biodegradable and non-toxic and have the goal of substituting, in the long run, petrochemical compounds. Nonetheless, in both cases, the industrial development of a fully microbial-based organic chemistry is far from being a present reality due to the high production costs and product variability.

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