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Sporopollenin-based bio-microcapsules as green carriers for controlled delivery of pharmaceutical drugs

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ABSTRACT

The production and design of innovative in-body microcarrier platforms for the controlled delivery of bioactive substances to predefined sites continue to hold substantial promise for biotechnology and medicine by increasing their therapeutic benefits. In this scope, plant pollen-based biocapsules, sporopollenin structures, have emerged as an alternative to synthetic ones due to their low-cost, highly uniform size distribution, resistance to physical and chemical conditions, and renewable green sources. Sporopollenin-based microcarriers, acting as a cargo and protective system, can be engineered to tune the biodistribution and therapeutic efficacy of encapsulated pharmaceuticals. Despite these benefits, the attained biocapsules directly from the plant, which have been the subject of research for nearly two decades, face several challenges and limitations, such as their availability without disrupting their layer integrity for all pollen species, dosage tuning, and the exact control of their responses on the immune system. Recent reports of successful oral administration seem, nevertheless, to bring them one step closer to clinical applications. Herein, we discuss the challenges, possible solutions for broadening natural resources and access to pollen, their further development towards the improvement of controlled release and prolonging the residence time in the intestinal lumen, and promising applications in the *in vivo* models and clinical trials, focusing on progress in biocapsule technology and the main events occurring along the way.

1. Relevance of the research on versatile microcarriers

The broad-spectrum interdisciplinary field of biotechnology turns challenges into opportunities, including the development of encapsulation and controlled release systems. With the progressive elaborations and discoveries in the field, notable advances have been achieved in the fabrication and engineering of micro-sized carrier systems for active pharmaceuticals, including drugs, enzymes, proteins, peptides, and DNA/RNA [1–6]. One of the aims of this field is to adopt green chemistry and green engineering principles to produce and/or non-toxic to humans and eco-friendly modify multifunctional assemblies. Even though this desired goal might be possible nowadays, it has narrow applications due to the specific chemistry requirements for their production. In addition to ensuring optimal bioavailability and biostability of encapsulated bioactive compounds [7], their delivery on targeted cells or tissues is also highly challenging owing to specificity. Encapsulated drugs, especially cytotoxic ones, must be delivered to the targeted area, so it is important to rationally design micro-carrier systems that can adjust the exposure time of the cell or tissue to the drug, along with ensuring that the right dose is released to a predefined area without causing serious side effects [8,9]. To control the encapsulation efficacy, but also the released dosage, its critical to accurately quantify the encapsulated drug or bioactive material in various media. While UV–Vis spectrophotometry, Fourier transformation infrared spectrophotometry,

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chromatographic, mass spectrometry, and fluorimetry methods are frequently used for this purpose, there are also works reporting advantages of electrochemical techniques for drug quantification, such as simplicity, ease of miniaturization, and high sensitivity [10,11].

Moreover, the delivery device must be able to adapt to the target application such as oral administration: with specificities to mask the unpleasant taste of pharmaceuticals, protect from the harsh conditions of the gastrointestinal tract and first-pass liver effects, and remain active when released from the capsulated form into the target site; for topical formulations: it should overcome low skin permeability, provide a longer delivery time at the applied skin site and increase drug absorption as well; and for intravenous administration: where the carrier systems may be easily recognized and cleared by the host immune system [9]. Other obstacles include maintenance in the bloodstream without sacrificing their effectiveness and delayed allergy [9]. Therefore, there is a need to design tailor-made micro-sized carriers considering the above mentioned factors. In fact, it would be desirable to combine multiple functional properties of delivery systems that provide site-specific targeting of active pharmaceuticals into a single carrier. Thus, these carriers could be programmed to predefined sites such as cells or tissues and more efficiently deliver pharmaceuticals to be used for several administration routes, without the need to redesign for each administration route. Although this concept is yet not realistic nowadays, progress in biotechnology could make it possible in a near future.

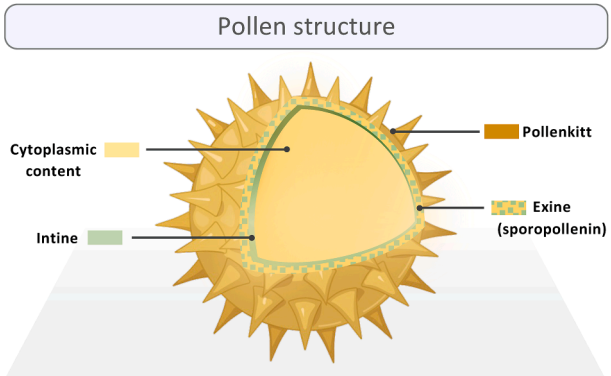
Many approaches, using natural and synthetic materials such as zeolites or polyacrylamide, have been explored for delivery systems using microencapsulation technology [12,13], especially for oral and topical administration, because they offer substantial advantages for drug delivery over conventional ones [14]. Current data demonstrate that natural polymers (alginate, chitosan cellulose, xanthan, etc.) are given priority over synthetic ones owing to their low potential toxicity and biodegradability in body fluids as well as biocompatibility characteristics [3,15–17]. However, polymer-based carrier systems face current barriers and limitations: dosage tuning, fabrication of uniform size microcapsules, production of acceptable quality and clinical shelf life, and such-like factors [9,18–20]. Pollen-based carriers, existing in naturally capsulated form, are another approach that has gained substantial attention in recent years to overcome these challenges [21]. Those are pollen grains obtained directly from plant pollen or from bee pollen pellets and belong to the natural biomaterials class. These biocapsules have shown strong potential as carriers for a variety of substances, including drugs, proteins, living cells, and oils ensuring an attractive, natural, and robust delivery platform due to their mechanical and chemical robustness, uniformity of size within each plant species, allergy-free, non-toxic, adjustable for drug encapsulation and delivery, and their easy availability from renewable sources [22]. Beyond these interesting features of biocapsules, the main concern is that they might cause allergic reactions, which can be overcome by removing their lipid and proteinaceous structures through a series of chemical treatments [23–25]. Nevertheless, it remains to be clarified whether biocapsules can be developed to have more functions and are worthy of the concept of multifunctional carriers in real life yet.

A deeper discussion is currently lacking on the technology of using plant-based green biocapsules as delivery platforms for active substances. Concerning this gap, this article discusses the different approaches to obtaining biocapsules, with particular focus on recent strategies for entrapping active pharmaceuticals into biocapsules, the future potential of these bio-carriers as effective delivery systems of pharmaceutical drugs, their suitability for specific needs, and associated challenges. Finally, we highlight the opportunities and future perspectives for biocapsules.

2. Plant-made biocapsules as green carrier systems

The production of identical micron-scale capsules is a longstanding aim in developing successful encapsulation and delivery strategies. While different fabrication approaches were developed in past decades, nature's creative capabilities have evolved over time to produce large quantities of elegantly sophisticated microcapsules with high constancy. Pollen micro-sized biocapsules are a clear example of this. In nature, pollen grains guarantee the reproductive capabilities of plants by protecting genetic materials from unfavorable conditions such as high temperatures, pathogenic microorganisms, UV radiation, prolonged dehydration, etc. [26–28]. This protection is provided by a robust pollen wall consisting of a bilayer arrangement with an inner layer (intine), and an outer layer (exine), as shown in Fig. 1. The exine is made up of sporopollenin, which is a cross-linked biopolymer with extreme durability, mechanical strength, and chemical stability, while the intine is composed of load-bearing cellulose/hemicellulose microfibers and pectin contributing to the physical robustness and elasticity of the pollen grain [19,29–31]. Pollen grains are also covered with an oily layer that exists on the exine wall. This substance, called pollenkitt, is a mixture of saturated and unsaturated lipids and lesser amounts of carotenoids, flavonoids, proteins, and carbohydrates and is linked with the ability of pollen to stick to the body of the insects during pollination [32]. The size (⌀2.5–250 μm) and sophisticated architecture of pollen grains can vary considerably depending on plant species, nevertheless, all those produced by a particular type of plant species are relative identical in size and shape [33]. This feature is a key point, as it represents a simple way of producing the same functional micron-scale biocapsules in large quantities.

The strategies for obtaining biocapsules, except for a few recent works [25,34,35], are achieved through the collection, processing, and separation of the desired pollen species from the plant. Unfortunately, these series of operations require high costs, experience, energy, labor, and long-term processing. An alternative solution to the fabrication of micro-sized capsules is the use of bee pollen. It stands out as a better option for companies or scientific studies to benefit from bees in the production of micro-sized capsules from bee pollen pellets, which is faster and a sustainable resource. Bees mix the pollen grains collected from the plants with their own secretions, allowing the pollen to moisten and become pellets (1-4 mm in size), which they transport on the rear legs to the hive [36]. This transformed product is called bee pollen and each pellet contains thousands of pollen grains [36], by and large from the same plant. All that is required to gather bee pollen is to place



Size	Shape	Ornamentation	Aperture type
Very small: 2.4–10 μm	Spheroidal	Echinate	Porus
Small: 11–25 μm	Cylindrical	Gemmate	Colpus
Medium-sized: 26–50 μm	Polygonal	Reticulate	Poroid
Large: 51–100 μm	Bi/tri-saccate	Croton pattern	Sulcus
Very large: 101–250 μm	Irregular	Lophate	Nanochannels

Fig. 1. The general structure of a pollen grain (from *Helianthus*). The scheme summarizes the main constituents of pollen and some of the morphological features widespread in various species in nature. All information was generated by analysis of the PalDat - Palynological Database (http://paldat.org).

specific collectors, pollen traps, at the entrance of the hive [37]. The bees do the rest of the work flawlessly, see Fig. 2. The next stage is to separate the obtained bee pollen pellets by color, and generally a single pollen pellet, collected at a specific time and place, is one colored load reflecting a homogeneous and monospecific pollen content [36].

The potential advantages of using bees to obtain biocapsules are enormous: collecting by a simple technique, cost-effective, time-saving, eliminating difficulties such as the use and management of special fields to produce biocapsules, and minimum energy input. Beside, using biocapsules derived from bee pollen pellets or directly from plants can overcome: (i) the need for different chemicals and high-cost equipment for encapsulation; (ii) problems like different designs and synthesis of microcapsules with suitable sizes and shapes for each application purpose; and (iii) toxic effects of synthetically produced microcapsules on the living organism.

Pollen grains are natural distribution vehicles that protect sensitive genetic material until they are received by female reproductive structures. The micro-sized capsule structure and ornamental architecture are common features across various plant species [30,38]. The openings located through the exine wall, are the first platform for transferring genetic material and feeding the sporoplasm with nutrients in a living spore. The two most evident pathways identified are (i) apertures (large portholes, ~1-2 μm in diameter), and (ii) multi-directional nano-structured channels (~40 nm for *Chenopodium album*) that allow water, nutrients, and signaling agents to be transported across the wall [31,39,40]. The presence of these micro-structured apertures and nano-structured channels can be explored for the artificial removal of the cytoplasmic content and its replacement with active substances, without damaging the wall structure of the pollen.

Instead of naming pollen-based microcapsules as sporopollenin exine capsules (SECs [41] and SpECs [42]), sporoderm microcapsules (SDMCs) [43], sporopollenin sporoderm microcapsules (S-SMCs) [20] or pollen grains (PGs) [23] merely by referring to their outer layer (exine), the term ''biocapsule'' seems more precise due to the potential usability of both layers of the pollen wall (inner and outer) in the design of the microcarrier systems. Biocapsules have been the subject of research in the scientific community because of its potential advantages over synthetic material-based microcapsules and large and renewable supply source, along with being biocompatible. The revealing of suitable loading and release techniques for pollen microcapsules and the idea that various pharmaceutical substances could be encapsulated in these biocapsules, have motivated the recently developed studies demonstrating their potential as ideal and developable carrier systems [19,22,27,31,44–48].

Despite the various papers on advances in this field, the present review focuses not only on recent achievements on the potential of biocapsules as low-cost delivery systems, but also on current challenges based on their efficiency and future perspectives to solve current problems.

3. Challenges and prospects in efficacious use of pollen-based biocapsules

Biomass is the most abundant renewable resource on Earth. The seventh principle of green chemistry states that chemicals from renewable sources, such as those from plants, must be preferred instead of those from petrochemical origin. Today, this principle forms the basis of intensive research on the (green) synthesis of chemical building blocks from biological feedstock of different chemical nature [33]. Through biological (anaerobic, fermentation, etc.) and/or thermochemical technologies (combustion, gasification, pyrolysis, etc.), renewable biomass can be transformed into biofuels, commodity chemicals, and new bio-based materials [49,50]. The switch to renewable biomass as a feedstock can afford an environmentally beneficial reduction in the carbon footprint of chemicals and liquid fuels. An additional benefit could be derived from the substitution of existing products by inherently safer alternatives with reduced environmental footprints, as can be exemplified by biocompatible and biodegradable materials [51]. Since chemical transformation processes from biomass could be required costly processes and energy for manufacturing new bio-based materials,

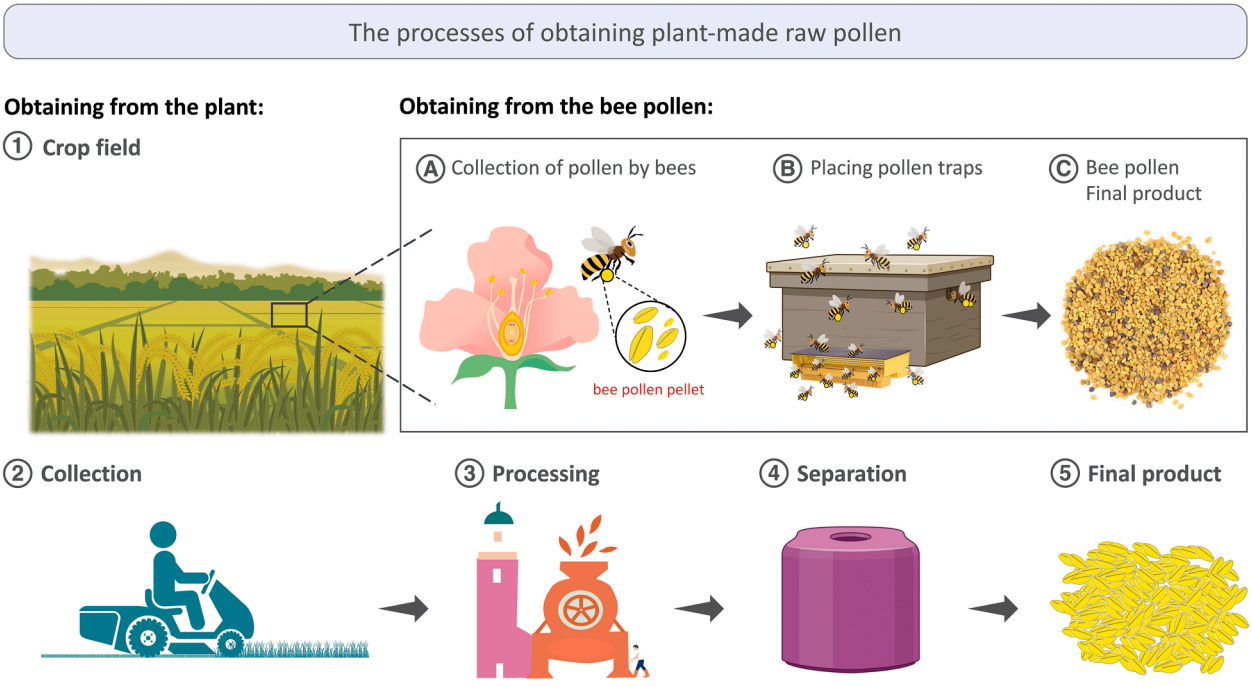


Fig. 2. Outline flow diagrams showing the availability of raw pollen from two sources for biocapsule production. (1) Greenhouse, crop field, or an area where a specific type of plant is abundant, (2) harvesting, (3) processing and storage of the crop, (4) separating the desired product from other plant parts, and (5) final product obtained directly from the plant, pollen. (A) Transport of pollen pellets to the hive by bees and collection of them by using pollen traps, (B) collection of pollen pellets carried by bees to the hive with pollen trap, and (C) the final product, bee pollen.

the research should aim to develop minimal energy and more cost-effective methods while preserving their chemical nature and transforming them into something useful and sustainable.

In particular, plant biomass, a lignin-carbohydrate complex composed of polymers of carbohydrates, lignin, proteins, pectins, and other components, are good sources that can be converted into bio-based products [52]. Moreover, plant biomass has different morphological fractions containing different complexes of cell wall components, e.g. reproductive cells. Although this is a challenge to achieve sustainable chemical production in biorefinery [52], its physical and chemical resistance may be desirable for different types of applications in biomaterials science [30]. The main success in bio-based materials fabrication versus petroleum-based is to select the correct biomass as raw material and exploit its components at maximum value.

Plant-made microcapsules, and particularly pollen, are a perfect starting point that fits the scenario described aforementioned, however, there are specific stages to address in order to explore their structure, first related to the removal of the pollen content and isolation of biocapsules and then to the upload of bioactive substances into it.

3.1. Isolation of biocapsules

The most common process to obtain usable biocapsules from pollen grains or bee pollen pellets follows two procedures sequentially: first, the pollen grains must be gradually exposed to solvents such as acetone and diethyl ether, several times, to remove oils, water-soluble carbohydrates, and pollenkitt [22,25,34]. Secondly, to remove the inner layer and cytoplasmic materials as well as to obtain allergy-free microcapsules, the pollen grains must be conditioned, for extended periods and elevated temperatures, in strong bases (such as 5% potassium hydroxide) for alkaline lysis, followed by an acidolysis step in 85% phosphoric acid [24,27,30,45], see Fig. 3. Those chemical treatments usually take place in a reaction flask under continuous heating and mechanical agitation. Between each chemical step, the residues are separated from the biocapsules, commonly by vacuum filtration [25]. Biocapsules are known for their resistance to chemical degradation, nevertheless, these series of processes can damage their morphological features [35,53] and peracetylate the alcoholic functionalities [33]. So, the optimization of the cleaning processes, while preserving their three-dimensional structure and functional properties, is an issue that requires major attention.

In general, the conventional method described above is a successful way to produce clean and intact pollen shells with a low level of residual protein content, from various pollen species. Gonzalez-Cruz and colleagues [54] showed, however, that optimization must sometimes be adjusted to the morphological structure of the pollen species. The authors evidenced that the conventional technique was successful with Lycopodium (*Lycopodium clavatum*) pollen, but failed for ragweed (*Ambrosia elatior*), however, this problem could be overcome by altering the sequence of alkali and acid purification steps.

The chemical treatments based on these conventional procedures employ noxious compounds, which are not *"green"* at all. Therefore, a chemical route that defines the optimization and development of green and easy isolation techniques for obtaining biocapsules is desired so that it would make most of the steps, from the source of the microcapsules to their functionalization and use, sustainable and safe.

Chiappe and colleagues [55] suggested that a cleaner approach could be done using ionic liquids such as 1-(4-sulfonic acid) butyl-3-methylimidazolium hydrogensulphate $[MIMC_4SO_3H][HSO_4]$. In the study, it was reported that microcapsules from *Populus deltoides* (eastern cottonwood) pollen grains can be purified without ruptures and flatting, beside, the ionic liquid could be reused. The other recent study, following a similar path, has stated that biocapsules could be isolated either by using ionic liquid (40% tetrabutylphosphonium hydroxide aqueous solution, TBPH) or lignocellulose-degrading enzyme cocktails after acidic pre-treatment (2% sulfuric acid) [35].

Even though pollen grains have an enormous variety in their sophisticated surface architecture, form, and size, the researched pollen species so far are rather limited. Investigations have mostly focused on

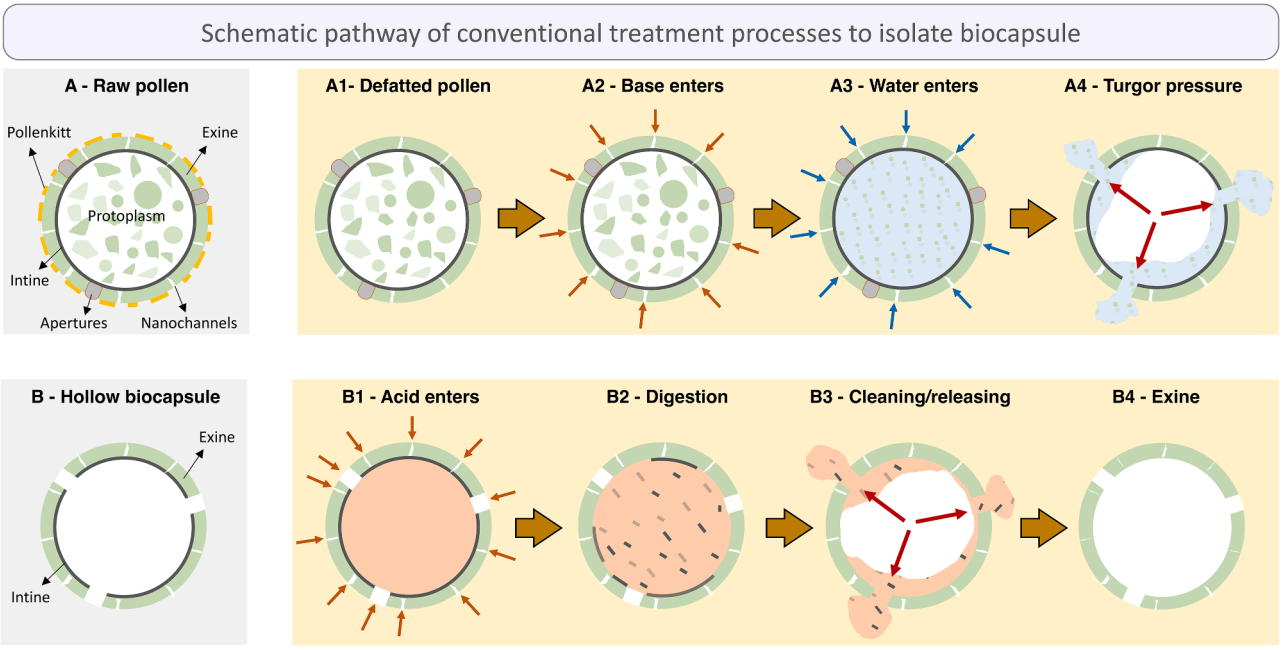


Fig. 3. Schematic of natural pollen grain (from *Cistus*) processing to isolate biocapsule in a conventional way. By applying defatting treatment to a raw pollen grain (A), the oily "pollenkitt" on the surface of the pollen is removed with acetone and diethyl ether (A1). The base enters the pollen grain through nanochannels and dissolves proteinaceous cytoplasmic molecules (A2). This creates an osmotic gradient, allowing more water to enter the pollen cavity (A3). In the inner cavity, the water causes a turgor pressure to build up and bursts the gaps, which are the thinner or absent regions of the exine layer, resulting in the evacuation of the contents (A4). The next step defines the purification of only the outer layer of the biocapsule consisting of exine and intine layers (B). Orthophosphoric acid enters the biocapsule (B1,) and dissolves the intine (B2). After a series of washing processes consisting of water, acetone, acid, base, water and ethanol (B3), the exine microcapsule emerges purely (B4).

the characterization of biocapsules of such as *Lycopodium clavatum* [22,24,30,31,56–60], *Helianthus annuus* L. (sunflower) [19,30,34,35,46,58,61,62], *Camellia Sinensis* L. (camellia) [20,22,25,30,34,58], *Typha angustifolia* (cattail), *Taraxacum officinale* (dandelion) [22,25,30,45,58], *Nelumbo nucifera* (lotus) [30,34,58] and *Ambrosia elatior* (ragweed) [23,40,54,62]. Given the diversity and the favorable properties of pollen biocapsules, which are durable biomaterials, it deserves further research to purify, characterize, functionalize, and identify suitable biocapsules, from different pollen species, to be employed in specific targets. As exemplified in Fig. 4, if the biocapsules are to be used for the delivery of orally active pharmaceuticals to the small intestine, obstacles like low adhesion properties and short residence time can be overcome by choosing a pollen type with a greater surface area to contact intestinal epithelial cells and with improved hydrophilic properties, which may result in a higher bioaccessible score [20].

Another issue to be highlighted is that the functional groups and surface charge of the outer wall of the biocapsules may change/functionalize with the chemicals applied during their purification [24]. Biocapsules shells and protein interactions during the uploading step could emerge undesirable and unpredictable effects, so it is crucial to understand these interactions to determine the immunogenicity and safety of capsules. Uddin and colleagues [63] describe this situation well: the outer surface of the biocapsules supported the formation of −COOH (cause neutral surface charge) in an acidic solution, while the biocapsules supported the formation of $–COO^-$ (cause negative surface charge) functional groups when placed in the basic solution, and thus different pH values could be used to change the surface charge of the shell. The study also revealed that in the analysis of the alteration of surface charge and the binding capacity of lysozyme and bovine serum albumin (BSA) model proteins, lysozyme adsorption onto biocapsules was mediated by electrostatic interaction between the negatively charged shell and the positively charged lysozyme, whereas negatively charged BSA failed to adsorb onto the biocapsule shells. In similar studies, it has been demonstrated that the outer layer of biocapsules isolated from ragweed pollen species adsorb lysozyme, ovalbumin (OVA) [64] and also chemokines (IL-8, MCP-1) [23], which are signal molecules secreted by cells.

Generally, all research on pollen-based microcapsules aims to isolate exin capsules directly by removing all components of pollen, however, the intine layer can also be explored from pollen grains and modified chemically. Fang and colleagues [65] described a simple way to isolate exine-free biocapsules after treating them with a sucrose solution supplemented with H_3BO_3, $CaCl_2$ (base medium), cellulase R-10, and macerozyme R-10, by gently grinding the droplets containing the pollen grains with a coverslip, at the appropriate pressure, to release pollen purified from the exine shell. The authors also emphasized that the isolation protocol did not damage the three-dimensional structure of the intine, and the protocol is reproducible. Although there is no direct evidence in the literature that intine or exine-intine may be specifically designed for the delivery of active pharmaceuticals, Fan and colleagues [30] produced microgel particles by converting pectin to pectate in the intine layer and stated that it could be employed as drug delivery system. Given the current advances in loading the active substances into biocapsules and delivering them to the desired site, it is worth emphasizing that the intine layer is a designable potential biomaterial that remains to be explored for such purposes. Cellulose and pectin, which constitute the structure of intine, pass into the intestines without being degraded in the stomach, here almost all the pectin is degraded by enzymes, while cellulose is fermented and partially digested owing to the short-chain fatty acids, hydrogen, carbon dioxide and methane produced by the intestinal microflora [66–68]. Considering the delivery of active pharmaceuticals by this route, the investigation of intine as a site-targeted drug carrier would be another step toward expanding the potential of biocapsules.

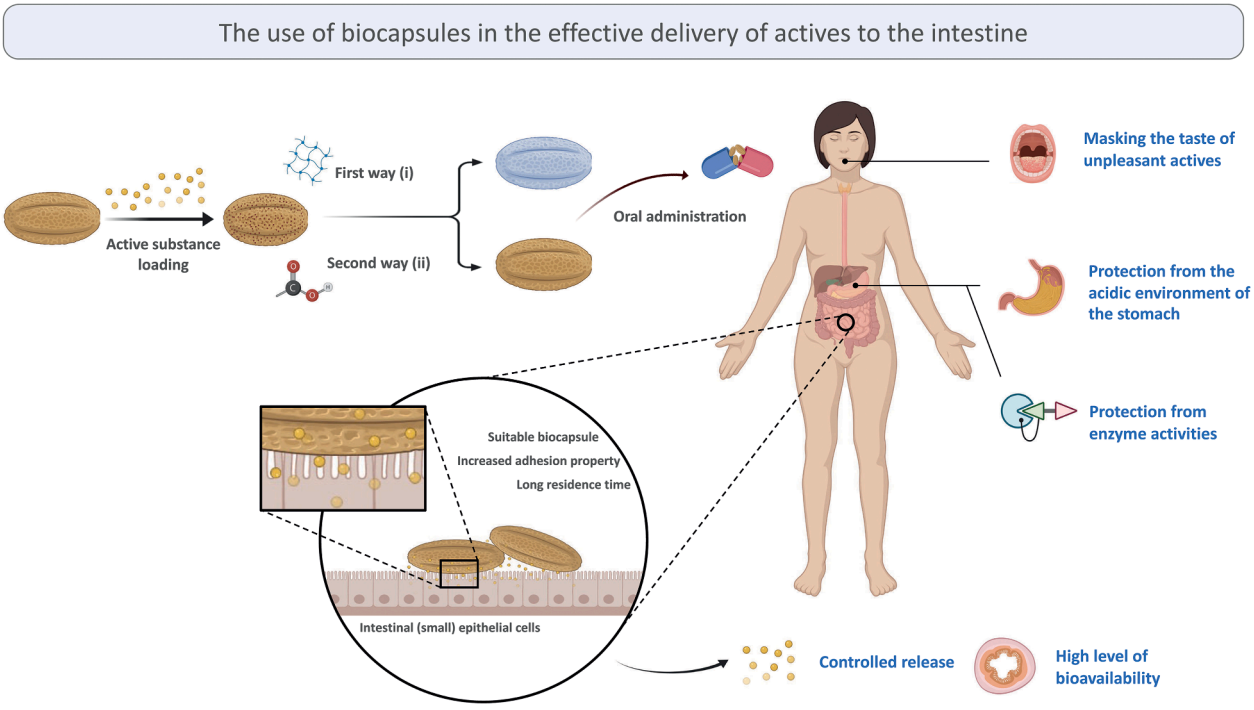


Fig. 4. The delivery mechanism of biocapsules (from *Arabidopsis*) for site-targeted active substances or oral vaccines. Hollow biocapsules as carrier systems: the first way (i) describes the coating of biocapsule with polymers such as alginate and eudragit, while the second way (ii) describes methods such as attaching substances to the exine surface, loading inside, or attachment of the matrix in which the substance is embedded to the inner surface of the biocapsule. After oral administration, it is aimed to prevent the premature release of active substance-loaded biocapsules throughout the gastrointestinal tract and to protect them as far as they reach. Beside, choosing the suitable biocapsule and designing it for the purpose ensures adhesion to the epithelial surface of the small intestine and prolonged release. Thus, a high bioavailability score is achieved in all cases.

3.2. Loading active substances into biocapsules

One way to load different types of active substances into biocapsules is the passive loading technique, where the biocapsules and the desired actives are mixed and loaded through the pores in the capsule wall, mostly by solvent diffusion, followed by gentle centrifugation, dialysis, or solvent evaporation to remove the unloaded molecules [69]. This procedure allows the encapsulation of substances such as pharmaceutical drugs [19,29] but also different kinds of low-viscosity lipophilic oils and waxes [70]. An exemplary study stated that biocapsules from *Lycopodium clavatum* were able to encapsulate oils up to a loading of 75%, while masking the unpleasant flavor of cod liver oil and sunflower oil at an encapsulation of less than 50% [71]. The solubility of the active substances may be a limitation during loading, since the solvent itself occupies part of the volume in the capsule, but higher loading can be achieved through a series of fills that accumulate progressively the active substances inside the capsule at each step [39].

Another commonly used loading technique is based on the use of a low vacuum to force molecules into the hollow space through suction-induced low pressure, Fig. 5 [31,69]. There is a driving external force here, hence it is expected to conclude in a relatively higher loading capacity compared to the passive loading technique [31,46]. Biocapsules purified from sunflower pollen species were loaded with bovine serum albumin (BSA) by passive and vacuum loading techniques and the result was that vacuum loading was 43.3% more efficient [19]. Nevertheless, considering the wide morphological diversity of biocapsules, it seems that remarkable results can be achieved for both techniques by matching a suitable biocapsule with an active substance and optimizing the techniques. To date, several substances such as drugs, proteins, dyes, and oils have been successfully loaded into biocapsules by vacuum loading [2,31,69,72].

The compression loading technique, another alternative, uses the compression of biocapsules to obtain a cylindrical tablet [46]. Here it takes advantage of the elastic property of the biocapsule wall: when the tablets are added to the solution containing the active substance, the capsule pores open and swell, thus allowing loading [39]. Previous reports stated that biocapsules are structurally stable without any damage due to compression [19,39]. This procedure is less preferred compared to passive or vacuum loading techniques. Even though, it is possible to load different substances into biocapsules with compression with a loading efficiency lower than the vacuum technique, but almost the same as the passive loading technique [19,31]. One of the most important applications reached with this technique was the successful encapsulation of living cells, namely probiotics, where *Lactobacillus casei* cells were loaded into *Lycopodium clavatum* biocapsules through the pores opened during compression [47].

For highly viscous solutions/suspensions, the centrifuge assisted loading technique is seen as an alternative, since it results in a better flow of solvent by taking advantage of the high centrifugation speed [39]. An important consideration here is that the centrifuge speed must

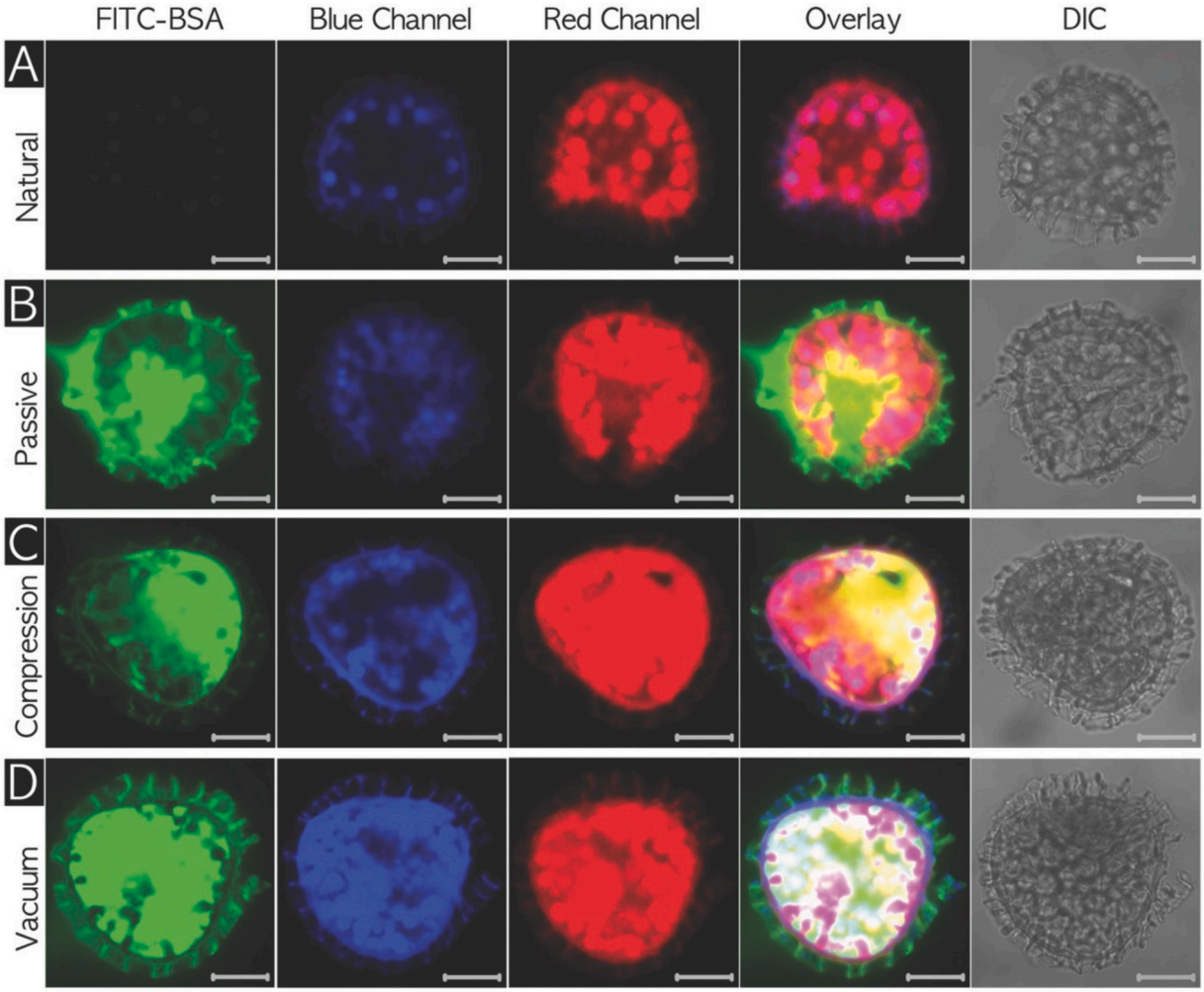


Fig. 5. Confocal laser scanning microscopy (CLSM) analysis of natural *Lycopodium clavatum* spores before and after macromolecule loading: (A) CLSM images of natural *Lycopodium clavatum* spores before BSA-loading. (B) BSA-loaded spores using the passive loading technique. (C) BSA-loaded spores using the compression loading technique. (D) BSA-loaded spores using the vacuum loading technique. Scale bars are 10 μm. Reproduced with permission from Ref. [31].

be optimized so that the biocapsules do not collapse and at the same time can be loaded with active substances. In the work conducted by Barrier and colleagues, the encapsulation of an alkaline phosphatase solution in glycerol, resulting in a 20% loading, could exemplify this technique [69]. Atalay and colleagues [41] also loaded BSA into biocapsules isolated from *Pinus, F. excelsior,* and *Tilia* pollen species by passive and centrifuge-based loading techniques. The study confirmed the higher centrifugal-assisted loading for all three pollen types compared to the passive one and linked it to the morphology and porous structure of the biocapsules.

Alternatively, it is possible to externally load active substances on the porous outer shell of some types of biocapsules, but in these cases, the loaded substance is directly exposed to the environment, what may not give the expected performance for a carrier system [19,39]. At the same time, due to the natural cycle of pollen and the ability of both the exine and intine layers to dehydration and hydration, it facilitates the ambient fluid to be drawn into the internal pollen cavity [39]. In this case, the loaded biocapsule may result in undesired leaks in body fluids, the decomposition of the loaded material in a strong acidic ambient like stomach acid, or the fact that it does not always accomplish the desired effect in conditions where a longer sustained release is required. There are quite limited studies reporting loading and releasing techniques that describe such difficulties, but it may be possible to overcome them by coating the outer layer of the biocapsule with different types of polymers [31,46] or by attaching a substance-embedded matrix to the inner surface of it. The study conducted by Lale and colleagues [40] is a representative attempt of this approach. In the study, BSA-embedded Eudragit L100-55 matrix was deposited on the inner surfaces of ragweed biocapsules, thus offering the minimal release of BSA without denaturing in simulated gastric acid fluid (pH = 1.2) and an additional mechanism of controlled release in the simulated intestinal fluid (pH = 6.8) during the first few hours after release, thanks to the solid walls of the biocapsules. In another study [73], coated biocapsules with carboxymethylpachymaran (CMP)/metal ion modification, as an oral delivery vehicle, were designed to preserve the activity of β-galactosidase and release it into the intestine. The designed CMP/3% $AlCl_3$ biocapsules showed remarkable performance in controlling release, with the residual activity of β-galactosidase at approximately 72% after 1 day of treatment. The increase in the number of such studies will provide valuable baseline information for future studies as well as direct the trends in the field toward *in vivo* and clinical studies.

4. Current advances in pollen-based biocapsules

The idea to explore pollen shells as carrier tools gained momentum in the early 2000s with research-oriented studies [74,75]. The isolation, functionalization, and detailed physical and chemical characterization of biocapsules have inspired their development and use as a multifunctional drug delivery platform. Beside being intra-body carrier systems that can be filled with various active substances, pollen biocapsules have also been the subject of intense research in other fields because of their mechanical and thermal durability, Table 1. One of the most explored examples is the use in separation technology [76] and in catalytic reactions [77] through the activating of their surfaces. Other recent reports reveal that it is also feasible to employ them in electrochemistry as electronic skin sensors [78], as well as in micron-structured bio-template [79]. Cojocaru and colleagues [80] investigated the detailed three-dimensional structure and features of *Pinaceae* pollen, and found that the wall structure of the pollen grain represents a solid biological nanofoam. This could encourage its use as an applied biomaterial, and also bio-template in the design of new materials. The development of biocapsules for different fields is an active research theme with recent reviews reporting the state of the art [81,82].

When comparing pollen-based biocapsule technology with traditional biocapsules, the most typical and promising property of the proposed biocapsules is the ability to tailor different features in a single structure and create novel versatile biomaterials. A representative case of this was shown by Wang and colleagues [91] when reported that spiky micromotors based on biocapsules isolated from sunflowers have the ability to both load and deliver BSA and absorb heavy metals without any additional modification. Thus, the advantageous properties of biocapsules are combined with their autonomous mobility, providing

Table 1
Applications of pollen-based biocapsules.

Source of pollen grain	Size	Research theme	Main finding	Refs.
Camellia sinensis	36.2 μm	Colloidal science and cellular applications	Ultraviolet-ozone treatment increased the proportion of surface elemental oxygen and ketones, leading to enhancedsurface hydrophilicity, enhanced particle dispersibility and cellular adhesion.	[83]
Camellia, Schisandra chinensis, lotus, rape flower and motherwort	Size range distribution: 15.0-30.0 μm	Energy storage systems	Carbon biocapsules microspheres were developed and synthesized materials showed attractive electrochemical performances for electrochemical double-layer capacitors with organic electrolyte, including large specific capacitance (250 F g^{-1} at a scan rate of 2 mV s^{-1}) and good cyclability (93.8% retention after 10 000 cycles).	[84]
Pinus rigida	-	Novel robust materials inspired by sporopollenin	Synthetic recapitulation of plant sporopollenin and analogues were presented.	[85]
Lycopodium clavatum	28.7 μm	Self-healing mechanism	Biocapsules containing rejuvenator agents were shown to promote self-heal cracks in asphalt pavements.	[86]
Helianthus annuus	-	Recyclable/reusable biocapsules-based paper for digital printing	It is demonstrated that the biocapsules-based paper exhibits high-quality printability, readability, and erasability, enabling its reuse.	[87]
Camellia sinensis L., *Typha angustifolia* L. and *Taraxacum officinale* L.	32.2, 19.5 and 27.1 μm, respectively	Physical and chemical degradation behavior in the human plasma	No significant deformation of the three-dimensional structure of the microcapsules occurred after 48 h of human plasma treatment.	[25]
Lycopodium clavatum	25.0 μm	Separation technology	Biocapsule-supported methylimidazolium was able to adsorb 84% of 2,4-dinitrophenol which is a water pollutant and could be reused for up to 5 cycles.	[88]
Rape pollen	Dimensions: ~20 × 40 μm	Bio-template	Hierarchically porous TiO_2 was prepared successfully using biocapsules as a biotemplate.	
Lycopodium clavatum, Camellia sinensis, Typha angustifolia and *Taraxacum officinale*	30.9, 31.8, 19.6 and 27.8 μm, respectively	Degradation behavior in the physiological conditions	Microcapsules showed different degradation scores in the gastrointestinal tract according to the species.	[22]
Pinus sylvestris L.	40.0 μm	Separation technology	ZnO-coated biocapsules were produced successfully and the product showed satisfying dye (Malachite Green) adsorption performance.	[89]
Juglans cinerea L.	27.0-33.0 μm	Catalytic reaction	The biocapsule-based Pd catalyst exhibited good catalytic behavior without any by-products.	[90]

a multifunctional platform for drug delivery and water purification applications. Studies on the isolation, development, and application of biocapsules have resulted in the publication of more than 150 papers in the last 5 years on the Scopus.

5. Promising applications in the active pharmaceutical ingredients

Tuning of pharmaceutical dosages of active substances, details of storage and processing, application form, and site to be delivered are crucial parameters to ensure appropriate efficacy for oral administration. While challenges, limitations, and points to investigate in designing biocapsules for microencapsulation of various substances exist, there are successful attempts in the literature reporting microencapsulation of active pharmaceuticals, Table 2. Several authors [23,64] administered biocapsules orally loaded with different proteins, either in the inner cavity or binding them to the outer layer of the biocapsule. Those can adhere to the intestinal epithelial layer, and thus aimed to obtain the systemic and high mucosal immune response, which is formed by B and T lymphocytes, antigen presenting cells, including dendritic cells, and specific epithelial and intraepithelial lymphocytes. Along with this achievement, it has also surprisingly reported the ability to generate an intrinsic immune response due to the complex architecture of the exine layer of biocapsules, from which protein and lipid constituents were removed.

For the microencapsulated oral vaccine delivery systems, Uddin and colleagues [64] loaded the biocapsules with OVA as a model vaccine antigen, after isolating chemical treated biocapsules with spiky (*Helianthus annuus* and *Ambrosia elatior*) and non-spiky (*Chenopodium album* and *Alnus glutinosa*) surface morphologies. The impact of the shell surface morphology of the biocapsules on the immune response, the duration of immunity, and the applicability of the delivery system across a diverse population of genetic backgrounds were sought. The results of the study demonstrated that in mouse serum immunized orally with the biocapsule-based OVA formulation, a strong and sustained anti-OVA IgG, IgG1, and IgG2a response was induced for 454 days after immunization. Along with this, the anti-OVA IgA response in the vaginal wash and saliva samples decreased by this time, however, significant levels of anti-OVA IgA were present in fecal samples. Beside, the study emphasized the efficacy of biocapsule surface morphology on the triggering of the immune response by selection criteria, emphasizing that species-specific biocapsule topographic features not only influence phagocytosis, but also OVA-specific antibody responses in serum, mucosal compartments, and bone marrow. An oral vaccine based on a chemically engineered biocapsule (*Ambrosia elatior*) has also been evaluated in the cell culture and mouse model to investigate the effect on the innate immune system after loading with OVA [23]. In the vaccinated mice with those biocapsules, mouse dendritic cells upregulated activation markers, namely CD40, CD80, CD86, and MHC class II molecules, and beside the interaction of biocapsules with Caco-2 cells induced the release of pro-inflammatory cytokines and chemokines, without compromising intestinal epithelial monolayer integrity.

Another successful study using spiky biocapsules isolated from sunflower pollen showed that the biocapsules in an *in vivo* melanoma (skin tumor) mouse model could effectively inhibit tumor growth by inducing cell apoptosis and reducing cell proliferation through near infrared light-triggered photothermal therapy [97]. Moreover, the authors underlined that sunflower biocapsules were still detected in tumor tissues up to 96 h after intratumoral injection. Referring to the fact that empty biocapsules can be filled with cancer drugs, the combination of encapsulated active drugs with near infrared light-triggered photothermal therapy for synergistic tumor therapy could be a potential application for biocapsules in the future. These data evidently remark that not only the loaded form of biocapsules is an active therapeutic weapon, but also their empty form can be employed as an active biomaterial for therapeutic intents.

Table 2
Key advances in the use of pollen-based biocapsules as carrier systems for health purposes.

Source of pollen grain	Size	Research theme	Main finding	Refs.
Lycopodium clavatum L.	28.0 μm	Controlled delivery system (model drug: Acetylsalicylic acid (aspirin)	The biocapsules showed slow release in the *in vitro* gastric ambient and rapid release in the intestinal ambient, indicating that the release is pH dependent.	[92]
Pinus, Fraxinus excelsior and *Tilia*	30-39, 18-22 and 34-35 μm, respectively	Delivery system (model protein: bovine serum albumin (BSA)	Morphology and porosity affected the encapsulation efficiency, and also in terms of loading capacity, *Tilia* biocapsules were the most efficient.	[41]
Pinus massoniana	62.2 μm	Delivery system (model protein: BSA)	Protein-loaded biocapsules coated alginate showed controlled release properties in the intestine.	[93]
Lycopodium clavatum	30.3 μm	Drug delivery system (model protein: BSA	Provided sustained release of the encapsulated active substance for up to 8 h at an *in vitro* adjusted gut ambient.	[31]
Corylus avellana	25.0 μm	Drug delivery system (model drug: Pantoprazole)	The pantoprazole was successfully loaded into microcapsules and exhibited a better release performance than the control under *in vitro* intestinal pH conditions.	[29]
Taraxacum officinale	29.3 μm	Drug delivery system (model protein: BSA)	Exine microcapsules were obtained without damaging their intrinsic architecture and BSA was loaded with high loading content and encapsulation efficiency.	[45]
Lycopodium clavatum and *Phoenix dactylifera*	28.0 and long-wide: 21-11 μm, respectively	Drug delivery system (model drug: Metformin (MTF)	In the *in vivo* study, the relative bioavailability of MTF-loaded biocapsule-alginate beads was 1.2 times higher than pure MTF.	[94]
Sunflower	37.9 μm	Drug delivery system (model protein: BSA)	Loaded microcapsules in alginate beads exhibited a controlled release	[19]

(*continued on next page*)

Table 2 (*continued*)

Source of pollen grain	Size	Research theme	Main finding	Refs.
			profile for up to 20 h.	
Helianthus annuus L.	30.3 μm	Drug delivery system (model protein: BSA)	BSA-loaded microcapsules protected the active until release at the targeted site and achieved complete release within 8 h.	[46]
Lycopodium clavatum	30.0 μm	Controlled delivery system (model drug: 5-fluorouracil)	Biocapsules coated with Eudragit RS100 for prolonged release of 5-fluorouracil performed better (up to 30 h) than those without.	[59]
Ambrosia elatior	–	Drug delivery system (model protein: BSA)	Protein-loaded microcapsules showed pH-dependent controlled release in the gastrointestinal tract (GIT).	[40]
Lycopodium clavatum	20.0 μm	Living cell delivery system	Encapsulated *Lactobacillus casei* cells exhibited higher viability compared to free cells in the GIT.	[47]
Sunflower	40.5 μm	Drug delivery system (model protein: β-galactosidase)	β-galactosidase was protected from the GIT conditions and the protease activity and successfully delivered to the intestinal ambient.	[2]
Lycopodium clavatum	28.0 μm	Delivery system (model active: Folic acid)	Microcapsules provided significant photoprotection for folic acid under UV and sunlight.	[18]
Betula pendula	25.0 μm	Drug delivery system (model drug: Imatinib mesylate)	Drug-loaded microcapsules acted on the WiDr human colon carcinoma cell line.	[95]
Ambrosia elatior	15.0-18.0 μm	Vaccine delivery system (model protein: Ovalbumin (OVA)	Strong systemic (anti-OVA IgG, IgG1, IgG2a, and IgA) and mucosal (anti-OVA IgA) immune responses that sustained for at least three months after vaccination.	[48]
Phoenix dactylifera L.	20.3 μm	Drug delivery system (model drug: Ibuprofen)	Drug-loaded microcapsules performed pH-sensitive release in the colon medium.	[44]
Helianthus annuus L., *Pinus taeda, Taraxacum officinale, Lycopodium clavatum, Typha angustifolia, Camellia Sinensis* L., *Nelumbo nucifera* and *Papaver rhoeas*	Size range distribution: 22.0-62.0 μm	Cancer drug delivery system (model drug: Doxorubicin)	Drug-loaded biocapsules resulted in a decrease in the viability of cells (MCF-7: breast cancer cell line) up to 41%.	[58]
Lycopodium clavatum	15.0 μm	Oral vaccine delivery system (model protein: OVA)	Biocapsules successfully filled with OVA for oral immunization elicited a potent anti-OVA IgG (systemic) and IgA (mucosal) antibody response.	[61]
Sunflower	–	Drug delivery system (model protein: β-galactosidase)	Microcapsule/3% $AlCl_3$ system showed controlled release with maximum residual activity of β-galactosidase at nearly 72%.	[73]
Sunflower	30.0 μm	Drug delivery system (nanoparticles: nobiletin/zein/tannin acid)	A controlled-release formulation of nanoparticles loaded-microcapsules was developed and demonstrated that the formulation can exist stably for at least 120 days at 4°C.	[96]
Lycopodium clavatum	20.0-25.0 μm	Drug delivery system (model drug: Diclofenac sodium (DIC)	Gastro-ulcerogenic results provided the potential for DIC-loaded biocapsules to significantly reduce gastric mucosal damage and protect submucosal cells in the rat stomach.	[72]

Other promising candidates include the microencapsulation of antibiotics. Macrolide-based erythromycin antibiotic is commonly used in the treatment of bacterial infections and is often prescribed as an alternative to patients with penicillin allergy [98]. However, low bioavailability, being unstable in stomach acid, and producing anhydroerythromycin, which is a more toxic by-product in an acidic ambient, are obstacles to its effective use as with most antibiotics and drugs [9,98,99]. Current advances show that biocapsules can be engineered to protect antibiotics from unfavorable environments and deliver them to the desired locations, with their optimum activity. Dyab and colleagues [100] loaded polypeptide bacitracin and erythromycin antibiotics into biocapsules (from *Lycopodium clavatum*) and applied them to an *in vitro*

and *in vivo* rat model, creating a therapeutic vehicle that could target delivery of oral administration of antibiotics. The results revealed a significant increase in the antibacterial fold activity of bacitracin and erythromycin-loaded biocapsules compared to pure antibiotics in the evaluation of antibacterial activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) human pathogenic bacterial strains. Furthermore, the study reported that the bioavailability of microencapsulated erythromycin was significantly enhanced, attributing to the mucosal adhesion of the biocapsules that allowed better release into the gastrointestinal tract in the rat, while being non-toxic to Caco-2 cells in the cytotoxicity assay.

A remarkable study [101] reported recently that 5-fluorouracil, which is used as a chemotherapy drug in the treatment of solid tumors of gastric, intestinal, and colorectal metastatic carcinomas, was loaded into biocapsules (from *Phoenix dactylifera*) and coated with Eudragit® RS-100 to prevent the discharge of 5-FU in the stomach and small intestine. The biocapsules were then administrated orally to rats for the treatment of colon cancer. The results of the study revealed that the 5-fluorouracil release rate in the *in vitro* simulated intestinal fluid of biocapsules coated with Eudragit® RS-100 was up to 24 h, while it was reduced to 12 h for uncoated ones. More importantly, the study reported that the coated biocapsules were protected from the upper gastrointestinal tract after oral administration, resulting in a 250-fold higher relative bioavailability of 5-fluorouracil in colon tissues, compared to uncoated biocapsules.

The first clinical evidence for the use of biocapsules isolated from *Lycopodium clavatum* L. pollen grains as an active substance delivery system was recently demonstrated by Diego-Taboada and colleagues [42]. The study reports the enhanced bioavailability of encapsulated vitamin D into biocapsules versus pure vitamin D, together with the efficient delivery mechanism of the actives to the intestine at the *in vitro, ex vivo*, and *in vivo* levels. The results revealed that encapsulated vitamin D exhibited 10-fold higher bioavailability than vitamin D alone in healthy volunteers, Fig. 6. This increase in bioavailability was supported by evidence based on a combination of the *in vitro, ex vivo* and *in vivo* data, and is due to the bioadhesion of biocapsules in the small intestine. The authors emphasized the potential of using biocapsules for high-dose vitamin D delivery in the treatment of vitamin D deficiency and the need for more comprehensive clinical studies.

6. Concluding remarks and perspectives

Current evidence demonstrates that plant-made pollen has been successfully isolated from its cellular constituents with high purity following a green path procedure. Beside their use in various research fields, biocapsules mediate bioavailability by ensuring stability to active pharmaceuticals by preventing degradation throughout the gastrointestinal tract. The knowledge gained in this field highlights that the applied processing/isolation protocols to remove cytoplasmic components and obtain hollow biocapsules from plant-based pollen or bee pollen, may affect the structural integrity of biocapsules walls and thus alter the bioavailability/stability balance of the therapeutic dose of formulation. The data also emphasize that the sophisticated architecture of the outer layer of biocapsules, called exine, may play an important role in the systemic and mucosal immune system by reducing or increasing the activities of immune responses influencing factors.

Pollen-based biocapsules offer significant advantages over synthetically produced ones due to their wide variety, robustness, amenability to modification, and flexibility in being designed for purposes. However, biocapsule technology brings with it some limitations that need to be overcome, such as their availability without disrupting their layer integrity, dosage tuning, and the exact control of their responses on the immune system. Researchers from different working groups around the world are persistently trying to solve these issues, while at the same time seeking solutions to overcome the difficulties that emerge in each new design, and these efforts have resulted in considerable progress in the field. Undoubtedly, the number of clinical studies using biocapsules is still reduced, but the first outputs reporting a 10-fold greater bioavailability of vitamin D loaded into biocapsules, without causing side effects, are promising.

Another important issue to be addressed is the standardization of biocapsules considering biosafety and efficacy requirements, properties/composition, and degrees of purity, isolation conditions, and reproducibility. This will enable the morphological, physicochemical properties, and biological activities of biocapsules to be cataloged under a single roof and will advance an important step towards easier selection and applicability for specific targets. Hence, applying the results of initial experiments, currently in mice, to larger animal models, would then cross the bottleneck in transitioning to clinical phases.

Realistically, progression in improving new pollen-based biocapsules therapeutic formulations and vaccines requires several research and development steps still need to be established along with safety protocols. These steps will be essential to allow biocapsule technology to become a real clinical therapeutic strategy.

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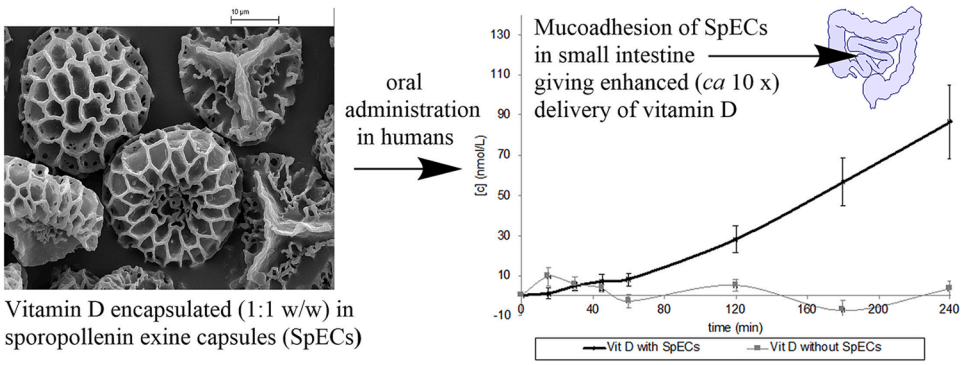

Fig. 6. The mean vitamin D2 serum level change in human volunteers ($n = 6$) over time obtained from oral administration of vitamin D2 alone or encapsulated in SpECs (sporopollenin exine capsules) extracted from *Lycopodium clavatum* pollen. Reproduced with permission from Ref. [42].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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