Arh. farm. 2023; 73: 404 – 422 *Review article/Pregledni rad*

Self-assembled carriers as drug delivery systems: current characterization challenges and future prospects

Ivana Pantelić[1,*], Tanja Ilić[1], Ines Nikolić[1,2], Snežana Savić[1]

[1] Department of Pharmaceutical Technology and Cosmetology, University of Belgrade – Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia

[2] Section of Pharmaceutical Sciences, Institute of Pharmaceutical Sciences of Western Switzerland – ISPSO, Biopharmaceutical Sciences, University of Geneva, CMU – Rue Michel Servet 1, 1211 Geneva 4, Switzerland

*Corresponding author: Ivana Pantelić, e-mail: ivana.pantelic@pharmacy.bg.ac.rs

Abstract

A review of recent publications reveals an increased interest in the so-called self-assembled carriers and their applicability in drug delivery via various routes of administration. Self-assembly denotes the process of rather spontaneous formation of ordered aggregates (sometimes under specific conditions – e.g., pH, temperature, ionic strength), via diverse interactions. This process, seen in many naturally occurring substances (polysaccharides, proteins, lipids), has inspired researchers to synthetize innovative self-assembling materials or combinations of existing ones. This paper provides a review of the recently investigated self-assembling materials and the carriers they form, often belonging to the sphere of pharmaceutical nanotechnology. Self-assembled carriers may provide enhanced stability, more efficient encapsulation and/or controlled delivery of active pharmaceutical ingredients. However, the diversity of geometries obtained (spheres, polyhedrals, ellipses, discs, porous structures, etc.) presents a significant characterization challenge, often requiring the application of several complementary techniques for proper evaluation of carrier size and morphology. Commonly utilized characterization techniques for investigating physico-chemical and certain biopharmaceutical properties are discussed, along with their advantages and disadvantages. Finally, the authors offer their critical opinion on the outlook of self-assembled drug carriers.

Key words: self-assembling materials, preparation methods, morphology assessment, sizing techniques, dialysis bag diffusion method

doi.org/10.5937/arhfarm73-46975

Introduction

When dispersed, amphiphilic molecules have long been known to self-assemble into diverse structures, if present in suitable concentrations (1). While low-molecular surfactants tend to form rather small and thermodynamically stable micelles, larger amphiphilic materials self-assemble into structures of a greater variety (2). Whereas micelles are mainly thought to provide or enhance solubilization of various pharmaceutical ingredients (either active ones or certain excipients), self-assembled structures with a higher level of organization may enhance their stability, encapsulation efficiency and/or provide modified or even targeted release. Research showed that, by combining or further modifying these so-called self-assembling materials, the geometry, stability and functionality of the obtained structured may be additionally tailored (3).

Self-assembly denotes the process of rather spontaneous formation of ordered aggregates (sometimes under specific conditions), via covalent, non-covalent, electrostatic or other interactions (4, 5). This process is not a recent invention. It is seen in many naturally occurring supramolecular architectures (building blocks), mediated by interactions at macroscopic, microscopic or nanoscale (4, 6).

A better understanding of the bonds stabilizing these self-assembling structures, along with the recognition of the variety of materials prospectively used and the design of novel ones, has led to increased interest in the fabrication of such systems. Using the PubMed® database, a review of the literature published in the last 5 years showed more than 18,000 papers containing *self* (and) *assembled* among the key words, while exactly 1,664 papers comprised *self* (and) *assembled* (and) *carriers* (7). On the other hand, patent search via the Espacenet base resulted in more than 60,000 matches, depending on the exact wording used (8). Apart from the apparent research interest, these numbers also reflect the actual applicability of self-assembled carriers.

Researchers seem to further exploit the self-assembling process, assessing its potential for a variety of materials. Promising polymeric representatives of synthetic, semi-synthetic and natural origin are being distinguished, their design and synthesis often inspired by certain polysaccharides, peptides/proteins or even lipids. The property common to these very different classes of substances is a high degree of structural regularity, obviously needed for the spontaneous formation of stable structures (9). In order to efficiently self-assemble, the molecules need to form direct interactions between certain functional groups. Hydrogen, π-π, van der Waals and hydrophobic bonding often initiate and/or maintain the self-assembly process, resulting in the formation of structures (10). The majority of the mentioned materials possess a certain degree of branching along the main backbone. This introduces additional attractive, but also repulsive electrostatic interactions to the system, usually due to, e.g., different isoelectric points, but also potentially influenced by ionic strength, pH and/or temperature. The aim is to obtain a formulation favoring a balance in these attractive and repulsive effects. Even some active pharmaceutical ingredients (APIs) show

marked amphiphilicity. This was recently utilized by Motlaq et al. (2) to obtain spontaneously formed vesicles of a phospholipid (1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC) and amitriptyline-hydrochloride by rather simple mixing and dilution.

This spontaneous assembling process may result in a variety of geometries. Although most commonly aiming for spherical particles, researchers have obtained other forms as well, allowing them some creativity in their description: polyhedrals, ellipses, elongated structures, discs, shells, nanocages, and core-shell structures, to name a few (9, 11). Unsurprisingly, when diverse nano-scaled structures are obtained, the term nanoplatform is often used (12). With persistent academic and industrial interest in nanosystems, self-assembled nanocarriers gained recognition as structures of a specific organization of building molecules, awarding them with unique physical properties. Among those properties, facilitated interaction with the physiological barriers or even living cells and their functions is sometimes mentioned (9). Contrary to other, more extensively studied nanocarriers (solid lipid nanoparticles, nanostructured lipid carriers) (13), another advantage of self-assembled formulations lies in the fact that their preparation seldom requires either specific/sophisticated equipment or elevated pressure (as, for instance, in high-pressure homogenization), high temperature or electric potential (as, for example, in electrospinning approaches) (9). Self-assembled nanocarriers are hence in line with the nowadays favored low-energy preparation processes (14-16), considering the fact that they tend to form spontaneously or with moderate energy consumption.

So far, findings show that self-assembled carriers may provide a greater versatility in administering APIs through diverse routes: oral, parenteral, (trans)dermal. For example, oral delivery of proteins and other challenging therapeutics is thought to benefit from such self-assembled carriers, both from the aspects of stability and efficacy (17-19). Self-assembled nanoparticles were also demonstrated to successfully load a wide range of actives, including small molecular pharmaceutical ingredients, DNAs, siRNAs and other larger structures (10, 20). Therefore, some of the mentioned self-assembled structures may serve as highly functional carriers for diverse actives: *i*) improving their efficient delivery (sometimes even offering targeted drug delivery to the diseased tissue), *ii*) stabilizing them (e.g., protecting them from light or harsh pH conditions of certain parts of the gastrointestinal tract), *iii*) improving other formulation aspects, making it more patient-friendly (e.g., preventing a burning sensation after parenteral administration of some chemotherapeutics) (21-23).

Therefore, although the formation of many extensively studied structures relies on self-assembling processes (such as micelles, liposomes, etc.) (24), this review will focus on other highly-ordered self-assembled structures potentially acting as drug delivery systems. Relevant self-assembling materials will be commented on, along with the commonly used preparation methods they require. Due to the specific construction of the self-assembled structures, advantages and disadvantages of the available

characterization methods for assessing their physico-chemical and in vitro biopharmaceutical parameters are discussed in more detail.

Materials showing self-assembly promising for drug delivery

A general overview of self-assembling materials and carriers made thereof for different aims and purposes may be found elsewhere (4, 7, 8). This section provides a more focused analysis of the self-assembling materials recognized for their role in forming drug carriers. However, one should search all potentially used wording for this type of materials and systems (e.g., self-assembly, self assembly, self-assembling, etc.).

As previously mentioned, some self-assembling materials form structures of versatile geometries, other than spherical ones. Careful structural analysis showed that such materials often possess a domain that does not self-assemble, but does not disturb the self-assembling process either. Such materials, usually peptide-based, are more likely to form nanofibers, sheets or some macroscopic structures, and show potential in tissue engineering, wound healing, etc. (4). Peptide/protein-based self-assembled carriers were intensively investigated in the last years (25, 26). This is chiefly due to the fact that peptide amphiphiles have altering structural regions with hydrophilic and hydrophobic properties, allowing them to spontaneously form tertiary nanostructures. These materials are generally considered to be biocompatible, and are easily modified with the addition of other substances (e.g., lipids, fluorescent markers) (26, 27).

The wealth of protein, polysaccharide and lipidic biomaterials should indeed be investigated for their potential in forming and stabilizing self-assembled systems. To name a few, whey protein, chitosan, levan, hyaluronic acid, and heparin, were tested native, in combination (e.g., protein-polysaccharide complexes), and after hydrophobic modifications (usually with octenyl-succinic anhydride, OSA) to enable the encapsulation of hydrophilic and lipophilic actives as well (28, 29). This is especially important considering the fact that a number of newly synthetized active ingredients are characterized with poor water solubility (30).

Some researchers showed more interest in polysaccharide-based self-assembly systems, such as chitosan. This is probably due to the natural origin of this cationic polysaccharide, accompanied with other favorable properties, such as biocompatibility and bioadhesion. Yuan et al. (31) used low molecular weight chitosan conjugated with lipoic acid (several degrees of substitution). After doxorubicin was loaded, the obtained formulation was tested and confirmed for certain redox-sensitivity.

A challenging oral delivery of insulin was also attempted via chitosan-based self-assemblies. Chellathurai et al. (18) tried to overcome this research challenge with spontaneously formed nanoparticles stabilized by electrostatic interactions between chitosan and insulin. The added value of considering self-assembled nanoparticles for this purpose is that their formation occurs rather fast and in mild conditions, not likely to degrade insulin in an early phase. Naturally, the obtained nanoparticles must offer prolonged insulin protection, shielding it from enzymes of the gastrointestinal tract. Although much is still needed for successful commercial oral delivery of insulin,

modified chitosan-based self-assembled carriers could indeed be one of the promising research avenues.

Among fructans, many types of inulins and levans showed promising self-assembling ability (32, 33). The diversity of their origin (plant, microbial) and structure (low and high molecular weight, with or without OSA modification) offer a wide range of applications. Pantelic et al. (34) investigated levan obtained from *Bacillus licheniformis* for prospective topical application, and found that self-assembled levan nanostructures generally complement the functional properties of topical emulsion systems.

Other natural-origin materials are also known for their self-assembly potential. Heparin, as one of the glycosaminoglycans, may be conjugated to some active ingredients. Lee et al. (35) linked doxorubicin to the carboxyl group of the unfractionated heparin, obtaining self-assembled nanoparticles for prospective anti-cancer therapy. Self-assembled liquid crystal cubic nanoparticles (stabilized with a combination of glyceryl monooleate, Pluronic F127 and HPMC) were investigated as delivery systems for highly lipophilic darifenacin-hydrobromide (an oral antimuscarinic investigated for overactive bladder treatment) (36).

Applying the self-assembling strategy, Zhao et al. (37) grafted a dendrimer in order to obtain spherical structures with several surface functionalities. This system was subsequently tested for transdermal delivery of isotretinoin, and showed an interesting release pattern: slower release was noted in normal tissue, while faster release occurred in lower pH tissues (inflammation model). Apart from satisfactory efficacy, good preliminary safety was shown as well, reflected in negligible skin irritation. Another group of researchers also designed a grafted self-assembling material, constituted of a peptide-decorated hyaluronic acid and fluorenylmethoxycarbonyl (Fmoc)-phenylalanine-phenylalanine-COOH peptide (38). These nanovesicles were investigated as prospective delivery systems for a hydrophobic active ingredient – curcumin. Detailed characterization revealed that curcumin was mainly located in the vesicles' walls, possibly mediated via π-π interactions.

A more comprehensive list of self-assembling materials is given in Table I.

Table I Selection of the investigated self-assembling materials, showing certain potential to form drug carriers

Tabela I Pregled materijala sklonih samoorganizovanju, sa potencijalom primene u formulaciji nosača lekovitih supstanci

Self-assembling material(s) used	Model active ingredient	Route of administration/application	Other excipients needed for preparation	Ref.
Polyoxyethylene esters of 12-hydroxystearic acid (Solutol® HS-15, BASF, Germany), D-α-tocopherol polyethylene glycol 100 succinate (TPGS, Sigma-Aldrich, USA)	Teniposide	Oral	Medium chain triglyceride, acetone, double distilled water	23
Copolymer of caffeic acid and chitosan (prepared *in house*)	Quercetin	Oral	Methanol	39
Poly(acrylic acid)-b-poly(N-isopropylacrylamide) and chitosan	Camptothecin	Oral	Acetate buffer pH 5.0, DMSO	40
Chitosan	Insulin	Oral	Acetic acid, sodium hydroxide, ethanol, Tris (hydroxymethyl) aminomethane	41
HPMC (10,000 Da), Poloxamer 407	Darifenacin-hydrobromide	Oral	Glyceryl monooleate, ethanol, propylene glycol	36
Sodium taurocholate	Berberine	Oral Intraperitoneal	Deionized water	5
1,2-dioleoyl-sn-glycero-3-phosphocholine	Amitriptyline-hydrochloride	(undefined)	NaCl solution	2
Polyethylene glycol (PEG)-coated poly D,L-lactic-co-glycolic acid (PLGA)	Memantine	Intrathecal	Poloxamer 407 (as pore-forming agent)	9
Low molecular weight chitosan-lipoic acid conjugates	Doxorubicin	Parenteral	N,N′-carbonyldiimidazole	31
Levan	Vancomycin	Parenteral	Na_2SO_4, distilled water	42
Dendrimer conjugate	Isotretinoin	Transdermal	4-dimethylaminopyridine, dicyclohexylcarbodiimide, toluene	37

Of course, when dealing with novel self-assembling materials, or new combinations/complexes of conventional ones, a set of suitable characterization methods should be applied for the evaluation of their inherent properties and compatibility with other planned formulation ingredients (e.g., testing of binary and ternary mixtures), prior to embarking on the formulation stage. These methods usually include but are not limited to Fourier transform infrared spectrometry (FTIR), nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC) and suitable chromatography techniques (9, 40).

Preparation methods for self-assembled carriers

Prior to the preparation step, the critical aggregation concentrations (CAC) of the selected self-assembling material needs to be determined. CAC represents the concentration of a self-assembling material able to form stable aggregates, usable for certain technological functions (43). As previously mentioned, depending on the nature of the applied material, various structures may be obtained (spheres, filaments, sheets, tubes, etc.) (11). CAC may be determined using several methods (relying on either particle size or fluorescence spectrophotometry) (39), or even predicted computationally (44).

Being liquid samples of generally low viscosity, self-assembled dispersions *per se* may not always be the appropriate dosage form. Hence, a number of researchers tried incorporating them into more conventional oral or topical drug dosage forms, especially those of the emulsion type (i.e., oral emulsions, cutaneous emulsions, creams) (29, 34) or gels (45). So far, the tested self-assembled dispersions were successfully combined with common excipients of the oily phase (such as medium chain triglycerides) and even sustained high shearing during homogenization (up to 24,000 rpm for 3 min) (29).

Naturally, preparation methods vary depending on the main stabilizer used. The majority of self-assembled formulations require simple preparation, involving only moderate energy input through mixing, either with a magnetic mixer, propeller, vortex, or ultra-sonication (46). Purified water or a suitable buffer are commonly used as the vehicles. Those self-assembling materials that are characterized with low critical aggregation concentrations (CAC < 0.1%) are often prepared as a more concentrated stock dispersion (e.g., 1%), and then further diluted (29). Another mode of self-assembled sample preparation involves the use of highly volatile solvents and their subsequent evaporation (using, e.g., a rotary evaporator) (5). Specifically, Zhang et al. (23) dissolved the needed active ingredients and excipients in acetone, and used reduced pressure for complete evaporation. The obtained thin film was subsequently dispersed with purified water at 37°C, while gently shaking. Fundamental approaches to preparation of self-assembled carriers are depicted in Figure 1.

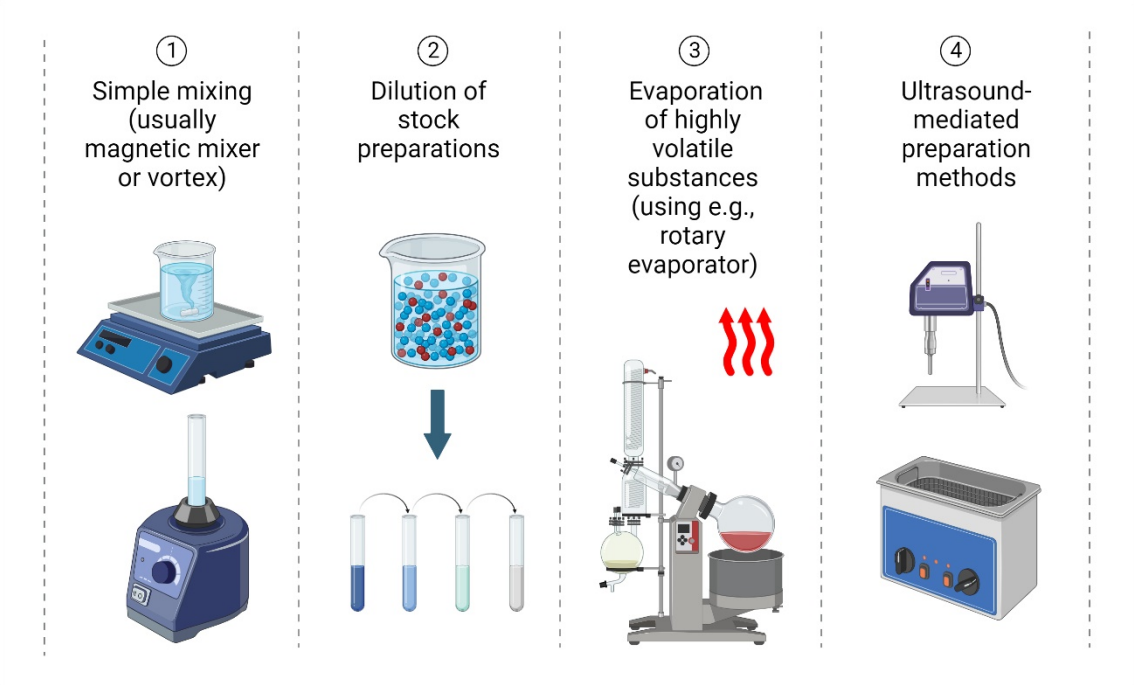

Figure 1. **Currently discerned preparation methods of drug carriers based on self-assembling materials (created with BioRender.com)**

Slika 1. **Osnovne metode izrade nosača lekovitih supstanci baziranih na prisustvu materijala sklonih samoorganizovanju (napravljeno pomoću BioRender.com)**

However, some advanced stimuli-responsive self-assembling materials need temperatures higher than the phase transition ones (temperatures above the cloud point) in order to properly self-assemble into stable nanoparticles. An example is the block copolymer of poly(acrylic acid-*b*-*N*-isopropylacrylamide). Dispersions of this copolymer were gently stirred at 45°C, resulting in self-assembled nanoparticles with both pH- and thermo-responsiveness preferred for controlled delivery of the encapsulated anticancer drugs (40).

Certain plant-origin self-assembling materials posed additional challenges. The solubility of walnut proteins is poor and pH-dependent (47). Hence, Lv et al. (48) used a pH-shifting preparation method (pH change from 7 to 12 (structural unfolding step) to 7 (refolding step)) to obtain walnut protein nanoparticles with solubility as high as 91%, but with a hydrophobic core. Curcumin was again selected as the model active ingredient, non-covalently quenched to the obtained nanoparticles.

Characterization methods available for assessing self-assembled carriers

Self-assembled structures have proven to be very challenging for characterization, often requiring several complementary characterization techniques to be applied. The following sections will provide an overview of the methodologies used so far,

highlighting their advantages and disadvantages when aiming for a comprehensive evaluation of a self-assembling formulation.

Morphology assessment

Whenever applicable, the morphology assessment of self-assembled structures should be carried out using a transmission electron microscope (TEM). Self-assembling materials having phosphate functional groups should be submitted through sample staining with 1% uranyl acetate, which may sometimes favor the visualization assessment (23). TEM images provide insight into the shape and uniformity of the nanoparticles. If crystals of the incorporated API are present, their distribution will also be apparent. However, whenever the loaded ingredient has a tendency to precipitate, its crystalline state should be assessed on an X-ray diffractometer. The absence of the API's characteristic crystalline diffraction peaks usually implies that self-assembled carriers successfully maintain it in the molecular state or amorphous form.

In some cases, high-resolution TEM (HR-TEM) is required to assess high density nanostructures. If the polymer is prone to certain secondary conformations, a non-routine circular dichroism (CD) spectroscopy is advised. CD is an absorption spectroscopy method advised for peptide/protein-based self-assemblies, based on the differential absorption of circularly polarized light (49). CD spectroscopy may also be used for assessing certain aspects of self-assembled structures' thermal stability (20). However, this technique is not routinely present in laboratories of formulation scientists.

Atomic force microscopy (AFM) is another highly informative technique applicable for self-assembled nanoparticles' visualization. This method is especially important for characterization of non-spherical carriers (50). Additionally, if needed, self-assembled nanoparticles may also be successfully loaded (labeled) with fluorescent dyes. Since self-assembled formulations are prone to certain changes over time, this opens the possibility of using so-called super-resolution microscopy techniques, potentially providing insight into the dynamics of the molecular exchange (11, 51).

Whenever dealing with larger or more complex self-assembling materials or mixtures thereof, small-angle neutron scattering (SANS) and/or small-angle x-ray scattering (SAXS) should be considered. These methods can reveal more detail on the obtained core-shell structures within the self-assembled sample, as well as whether transitions to structures of other geometries are likely to happen in time (2).

Although this review mainly focuses on polysaccharide and protein-based self-assembly systems, self-assembled lipid structures may also have many potential applications, including being carriers for water-insoluble or water-sensitive actives. Recently, Bryant et al. (52) investigated phytantriol (a non-ionic lipid) self-assembly with and without water, choline-chloride and urea. Such systems tend to form complex phases (in this case cubic and inverse hexagonal phase were dominant) prone to temperature-related phase transitions. Hence, characterization techniques such as SAXS and cross polarized optical microscopy are indispensable for understanding these highly ordered structures.

However, the fragile balance between the aggregation and disaggregation of the assembling molecules may easily be disturbed during the morphology assessment, sometimes providing false notions on the geometry of the obtained particles. Therefore, the complexity of the investigated self-assembled nanoplatforms always requires a combined use of characterization methods. In such cases, morphology assessment is closely discussed with suitable sizing technique(s) (33, 35).

Sizing techniques

Size and size distribution assessment is another characterization challenge for self-assembled carriers. Dynamic light scattering (DLS) is a commonly used technique, available in a majority of interested formulation scientists' laboratories. However, whenever dealing with non-spherical nanostructures, DLS provides an unsatisfactory size approximation and distribution results. Ideally, PDI values below 0.2 are favored and indicate that nanoparticles of satisfactory uniformity are obtained. Nevertheless, when aiming for a more comprehensive characterization of self-assembled nanocarriers, an additional sizing technique should be applied whenever available, preferably those providing information on a single nanoparticle level, such as Nanoparticle Tracking Analysis or Tunable Resistive Pulse Sensing (53, 54). Unfortunately, such techniques are not applied routinely.

Another disadvantage of the DLS approach is in the common need to dilute the sample prior to measurement (e.g., 1:10), with or without additional vortexing (36). This may disturb the reached balance among the attractive and repulsive forces, and lead to marked fluctuation of the scattered light and therefore false results. The medium for dilution may also influence the obtained sizes and zeta potentials, especially if it contains electrolytes. When opting for peptide/protein-based self-assembly structures, less positively charged nanoparticles (e.g., with zeta potential in the range +5 to +20 mV) are considered a safer option, with generally satisfactory results of cytotoxicity (usually confirmed by MTT and/or LDH) assays (20).

Other relevant physico-chemical characterization techniques

Not neglecting the amphiphilicity of the self-assembling materials, surface tension should be determined for certain self-assembled formulations, especially when anticipating that this property may interact with other needed excipients, or further influence drug loading efficiency and/or subsequent steps leading to drug delivery (release, permeation, penetration, etc.). Good examples of studies where assessing surface tension of the obtained self-assembled samples was indispensable are those dealing with ophthalmic and pulmonary drug delivery (55, 56).

Whenever formulations aim to serve as carriers, parameters such as encapsulation efficiency (EE) should be determined. Encapsulation or entrapment efficiency represents the amount or the concentration of the active ingredient successfully loaded into the formulated carrier, assuming the free active ingredient remains in the supernatant after sample centrifugation. Thus, the optimal combination of centrifugation rate/time

(parameters used by researchers span from 3000 x g / 5 min to 12,000 rpm / 10 min), filter diameter (e.g., 10,000 Da Milipore filter) and the quantification method (sometimes UV-Visible spectrophotometry, but more often the HPLC method with defined column type, mobile phase composition, flow rate, etc.) need to be found for this (ultra)filtration approach to be reliable (9, 23, 57).

Overall stability of the obtained self-assembled formulations should also be assessed, following a protocol most suited to the final drug dosage form, but usually comprising centrifugation, heating, cooling and/or freeze-thaw cycles (5). Thermal stability is meticulously investigated when self-assembled structures are developed as carriers or adjuvants for vaccine formulations (58). Nevertheless, a number of studies in the field publish only preliminary stability results, relying on up to 1 month storage (40, 59). Whenever applicable, internationally accepted recommendations should be acknowledged, such as the ICH Q1A (R2) guideline on Stability Testing of New Drug Substances and Products (60). However, throughout the stability study, specific attention should be given to monitoring the sensitive balance between aggregation and disaggregation of the self-assembling formulations.

In vitro testing of biopharmaceutical properties

The intended route of application/administration usually determines the exact manner in which the in vitro release profiles of APIs loaded into self-assembled carriers should be determined. Considering the fact that the majority of the developed self-assembled formulations are liquid preparations, containing particles of nanometric sizes, the dialysis method (using dialysis membrane sacs/bags in a variety of molecular weight cut-offs, e.g., 10,000-14,000) is favored by the researchers, varying the type of the buffer solution used (e.g., 0.1 N HCl simulating gastric juice at pH 1.2, or phosphate buffers (PBS) of several pH values). These conditions are summarized in Table II.

Striving for more biologically relevant membranes, some researchers tested the ability of self-assembled formulations to permeate the intestinal membrane (e.g., from adult Albino rabbits' intestine) (36). Although scarce, such ex vivo studies may serve as a good transition from in vitro results to in vivo settings.

The formulation of peptides and proteins remains a constant challenge, irrespective of the intended route of administration. However, certain researchers tried to utilize their natural amphipathy, and hence, tendency to self-assemble into nanostructures (10). This has resulted in a number of functional peptides and proteins acting as either actives with enhanced efficacy and/or stability, or biocompatible excipients. However, these self-assembled structures based on peptide amphiphiles share a common property – high susceptibility to microenvironmental changes. Nevertheless, this characteristic may not be taken as a disadvantage *per se*, but further explored for, e.g., targeted membrane fusion or other modes of efficient delivery. Proof of such processes is usually assessed within cell uptake studies. Although cell-based and in vivo studies go beyond the scope of this manuscript, considering the inherent ability of self-assembled nanoparticles to promote membrane fusion, certain fluorescence microscopy studies are advised in order to

investigate the subcellular localization of labelled nanoparticles (28). Barel et al. (20) applied fluorescence-activated cell sorting (FACS) for Alexa 488-labelled bDNA nanoparticles on HCT-116 cells to compare the degree of bDNA cell internalization with and without the self-assembled nanocarrier.

Table II An overview of the experimental conditions applied within the dialysis bag diffusion method

Tabela II Pregled eksperimentalnih uslova primenjenih kod metode sa dijaliznim vrećicama

Membrane type	Membrane cut-off	Preconditions	Media	SA formulation type	Reference
Cellulose membrane (HIMEDIA, India)	12,000-14,000 Da	Hydrated overnight in dissolution media	Acidic buffer pH 1.2; Phosphate buffers pH 6.8 and 7.4	Memantine-loaded poly (lactic-co-glycolic acid) (PLGA), PEG 35000 and Pluronic F-127 self-assembled nanoscaffolds	9
(unspecified)	12,000-14,000 Da	(unspecified)	HCl buffer pH 2.0; Phosphate buffers pH 6.0 and 7.4	Camptothecin-loaded dual pH/thermos-responsive poly(acrylic acid-b-N-isopropylacrylamide nanoparticles	40
SpectraPor® (Repligen, USA)	12,000-14,000 Da	Hydrated for 1h in distilled water to remove preservative traces	Phosphate buffer pH 7.4	Darifenacin-loaded liquid crystal cubic nanoparticles	36
SpectraPor® (Repligen, USA)	3,500 Da	(unspecified)	Phosphate buffer pH 7.4 with 1% polysorbate 80	Paclitaxel-loaded poly(D,L-lactic-co-glycolic acid) (PLGA) and poly(L-lactic acid) (PLA) nanoparticles	61

Current challenges and future prospects

Although much is known of the supramolecular levels of organization within self-assembled systems, recent creativity in the design and synthesis of novel self-assembling materials or hybrids of existing ones, opens new research questions. In their recent paper, Meng et al. (22) suggest that studying pressure-structure-property relationships may improve the collective knowledge of diverse nanoparticles, including self-assembled

structures. In addition, a lot is expected from peptide/protein-based self-assembled delivery systems. Even smaller peptides were shown to successfully self-assemble into spheres or fibers, depending on the exact nature of N-terminus modifications (65). The importance of the termini modifications was reported for materials based on certain cellulose alomorphs as well. Applying that concept, Hribernik et al. (66) managed to obtain both elongated and square-like structures. On the other hand, reflectin-based peptides tend to assemble into higher ordered structures such as hydrogels, films or even certain optically active biomaterials (67), but full understanding of their potential is yet to come. Furthermore, the introduction of additional properties to the obtained self-assembled carriers, such as, e.g., magnetic character, requires certain advanced levels of characterization (62). Still, due to the very diverse materials used, some self-assembled carriers already face a challenging scale up from laboratory levels (24). Moreover, certain self-assembled nanosystems proved able to be further organized into complex three-dimensional structures with tunable porosity (being equipped with compartments and corridors) (6). Such systems may provide more possibilities in improved stabilization, more efficient entrapment and/or controlled delivery of active ingredients.

Further, structural integrity of the formed self-assemblies and consequently their stability need to be properly investigated by a rational number of reliable characterization techniques. In this vein, interested formulators would benefit from a robust decision tree, guiding them stepwise through necessary characterization techniques. Nevertheless, this field is witnessing considerable progress, often linking researchers from quite diverse fields of expertise.

Finally, some authors go so far as to speculate self-assembled systems to be promising carriers for oral delivery of highly demanding active ingredients, such as antibodies (63). However, such notions still require more profound in vivo data substantiation. Nevertheless, it appears that a lot is expected from self-assembled peptides, not only for the delivery of drugs, but vaccine antigens as well (64). Naturally, adjuvant functionality substantiation would require additional testing.

Conclusion

The self-assembly processes are being increasingly exploited, due to the plethora of self-assembling materials and the fact that their preparation generally consumes less energy, making them attractive for the next generation, eco-conscious pharmaceutical industry. Although the stability of the API/self-assembled carrier association is of utmost importance for prospective drug delivery application, the exact mechanism of their dissociation is as important, but often hard to elucidate. Additionally, further research has yet to show the scale-up capabilities of each of the presented self-assembled formulations as prospective drug delivery systems. The fact that the transformation of self-assembled nanoparticles may be tuned to certain environmental triggers (e.g., pH, temperature, redox environment) offers a new range of possibilities, labelling them as ‘smart’ vesicles capable of achieving a degree of stimuli-responsiveness in vivo. Such formulations are envisioned for controlled drug release or promoting drug delivery and subsequent

accumulation into the diseased tissue. However, their comprehensive characterization often requires a wider set of techniques. Hence, introducing certain computational approaches and combining them with the existing experimental ones may facilitate the selection of self-assembling materials optimal for drug delivery purposes. Nevertheless, the wealth of self-assembling biomaterials opens unlimited possibilities for their application, modification and/or combination/complexation, as a platform for innovative materials in drug development.

Acknowledgements

This research was funded by the Ministry of Science, Technological Development and Innovation, Republic of Serbia through Grant Agreement with the University of Belgrade – Faculty of Pharmacy No: 451-03-47/2023-01/ 200161.

References

1. Taylor K, Aulton M, editors. Aulton’s Pharmaceutics - The Design and Manufacture of Medicines, 6th edition. London: Elsevier; 2022; 394 p.
2. Motlaq F, Gedda L, Edwards K, Doutch J, Bergström LM. Spontaneous Formation of Ultrasmall Unilamellar Vesicles in Mixtures of an Amphiphilic Drug and a Phospholipid. Langmuir. 2023;39(32):11337-44.
3. Northrop B, Zheng Y-R, Chi K-W, Stang P. Self-Organization in Coordination-Driven Self-Assembly. Acc Chem Res. 2009;42(10):1554–63.
4. Kumar P, Pillay V, Modi G, Choonara YE, du Toit LC, Naidoo D. Self-assembling peptides: implications for patenting in drug delivery and tissue engineering. Recent Pat Drug Deliv Formul. 2011;5(1):24-51.
5. Qin Z, Li M, Cheng J, Huang Z, Ai G, Qu C, et al. Self-Assembled Nanoparticles Combining Berberine and Sodium Taurocholate for Enhanced Anti-Hyperuricemia Effect. Int J Nanomedicine. 2023;18:4101-20.
6. Tanaka H, Dotera T, Hyde S. Programmable Self-Assembly of Nanoplates into Bicontinuous Nanostructures. ACS Nano. 2023;17(16): 15371-8.
7. PubMed [Internet] [cited 2023 Sep 20]. Available from: https://pubmed.ncbi.nlm.nih.gov/.
8. Espacenet [Internet] [cited 2023 Sep 20]. Available from: https://worldwide.espacenet.com/.
9. Rani V, Verma R, Kumar K, Chawla R. pH-influenced self-assembled stealth nanoscaffolds encapsulating memantine for treatment of Alzheimer's disease. J Biosci. 2023;48:31.
10. Song Z, Chen X, You X, Huang K, Dhinakar A, Gu Z, Wu J. Self-assembly of peptide amphiphiles for drug delivery: the role of peptide primary and secondary structures. Biomater Sci. 2017;5(12):2369-80.
11. Hendricks MP, Sato K, Palmer LC, Stupp SI. Supramolecular Assembly of Peptide Amphiphiles. Acc Chem Res. 2017;50(10):2440-8.

12. Liang J, Li L, Tian H, Wang Z, Liu G, Duan X, et al. Drug Repurposing-Based Brain-Targeting Self-Assembly Nanoplatform Using Enhanced Ferroptosis against Glioblastoma. Small. 2023;e2303073. doi: 10.1002/smll.202303073.
13. Müller RH, Radtke M, Wissing SA. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. Adv Drug Deliv Rev. 2002;54(1):S131-55.
14. Gledovic A, Janosevic Lezaic A, Nikolic I, Tasic-Kostov M, Antic-Stankovic J, Krstonosic V, et al. Polyglycerol Ester-Based Low Energy Nanoemulsions with Red Raspberry Seed Oil and Fruit Extracts: Formulation Development toward Effective In Vitro/In Vivo Bioperformance. Nanomaterials. 2021;11(1):217.
15. Grijalvo S, Rodriguez-Abreu C. Polymer nanoparticles from low-energy nanoemulsions for biomedical applications. Beilstein J Nanotechnol. 2023;14:339-50.
16. Nikolic I, Mitsou E, Pantelic I, Randjelovic D, Markovic B, Papadimitriou V, et al. Microstructure and biopharmaceutical performances of curcumin-loaded low-energy nanoemulsions containing eucalyptol and pinene: Terpenes' role overcome penetration enhancement effect? Eur J Pharm Sci. 2020;142:105135.
17. Chapa-Villarreal F, Miller M, Rodriguez-Cruz J, Pérez-Carlos D, Peppas N. Self-assembled block copolymer biomaterials for oral delivery of protein therapeutics. Rev Biomaterials. 2023;300:122191.
18. Chellathurai M, Yong C, Sofian Z, Sahudin S, Hasim N, Mahmood S. Self-assembled chitosan-insulin oral nanoparticles - A critical perspective review. Rev Int J Biol Macromol. 2023;243:125125.
19. Xia D, Hu C, Hou Y. Regorafenib loaded self-assembled lipid-based nanocarrier for colorectal cancer treatment via lymphatic absorption. Eur J Pharm Biopharm. 2023;185:165-76.
20. Baral B, Panigrahi B, Kar A, Tulsiyan KD, Suryakant U, Mandal D, Subudhi U. Peptide nanostructures-based delivery of DNA nanomaterial therapeutics for regulating gene expression. Mol Ther Nucleic Acids. 2023;33:493-510.
21. Cheng J, Zhao H, Li B, Zhang H, Zhao Q, Fu S, et al. Photosensitive pro-drug nanoassemblies harboring a chemotherapeutic dormancy function potentiates cancer immunotherapy. Acta Pharm Sin B. 2023;13(2):879-96.
22. Meng R, Hao S, Sun C, Hou Z, Hou Y, Wang L, et al. Reverse-QTY code design of active human serum albumin self-assembled amphiphilic nanoparticles for effective anti-tumor drug doxorubicin release in mice. Proc Natl Acad Sci. 2023;120(21):e2220173120.
23. Zhang Z, Ma L, Jiang S, Liu Z, Huang J, Chen L, et al. A self-assembled nanocarrier loading teniposide improves the oral delivery and drug concentration in tumor. J Control Release. 2013;166(1):30-7.
24. Kansız S, Elçin YM. Advanced liposome and polymersome-based drug delivery systems: Considerations for physicochemical properties, targeting strategies and stimuli-sensitive approaches. Rev Adv Colloid Interface Sci. 2023;317:102930.
25. Gomes A, Sobral PJDA. Plant Protein-Based Delivery Systems: An Emerging Approach for Increasing the Efficacy of Lipophilic Bioactive Compounds. Molecules. 2021;27(1):60.
26. Llanos MF, Gómara MJ, Haro I, López ES. Peptide Amphiphiles for Pharmaceutical Applications. Curr Med Chem. 2023. doi: 10.2174/0929867330666230408203820.

27. Staii C. Conformational Changes in Surface-Immobilized Proteins Measured Using Combined Atomic Force and Fluorescence Microscopy. Molecules. 2023;28(12):4632.
28. Duan S, Xia Y, Tian X, Cui J, Zhang X, Yang Q, et al. A multi-bioresponsive self-assembled nano drug delivery system based on hyaluronic acid and geraniol against liver cancer. Carbohydr Polym. 2023;310:120695.
29. Han L, Zhai R, Hu B, Yang J, Li Y, Xu Z, et al. Effects of Octenyl-Succinylated Chitosan-Whey Protein Isolated on Emulsion Properties, Astaxanthin Solubility, Stability, and Bioaccessibility. Foods. 2023;12(15):2898.
30. Ilić T, Đoković JB, Nikolić I, Mitrović JR, Pantelić I, Savić SD, Savić MM. Parenteral Lipid-Based Nanoparticles for CNS Disorders: Integrating Various Facets of Preclinical Evaluation towards More Effective Clinical Translation. Pharmaceutics. 2023;15(2):443.
31. Yuan Y, Wang Z, Su S, Mi Y, Li Q, Dong F, et al. Redox-sensitive self-assembled micelles based on low molecular weight chitosan-lipoic acid conjugates for the delivery of doxorubicin: Effect of substitution degree of lipoic acid. Int J Biol Macromol. 2023;247:125849.
32. Jiménez-Sánchez M, Pérez-Morales R, Goycoolea FM, Mueller M, Praznik W, Loeppert R, et al. Self-assembled high molecular weight inulin nanoparticles: Enzymatic synthesis, physicochemical and biological properties. Carbohydr Polym. 2019;215:160-9.
33. Lee JS, Park E, Oh H, Choi WI, Koo H. Levan nanoparticles with intrinsic CD44-targeting ability for tumor-targeted drug delivery. Int J Biol Macromol. 2023;234:123634.
34. Pantelić I, Lukić M, Gojgić-Cvijović G, Jakovljević D, Nikolić I, Lunter DJ, et al. Bacillus licheniformis levan as a functional biopolymer in topical drug dosage forms: From basic colloidal considerations to actual pharmaceutical application. Eur J Pharm Sci. 2020;142:105109.
35. Lee J-H, Yang S-B, Lee J-H, Lim H, Lee S, Kang T-B, et al. Doxorubicin covalently conjugated heparin displays anti-cancer activity as a self-assembled nanoparticle with a low-anticoagulant effect. Carbohydr Polym. 2023;314:120930.
36. Farag MM, El-Sebaie W, Basalious EB, El-Gazayerly ON. Darifenacin Self-assembled Liquid Crystal Cubic Nanoparticles: a Sustained Release Approach for an Overnight Control of Overactive Bladder. AAPS PharmSciTech. 2023;24(5):120.
37. Zhao T, Zhou M, Wu R, Wang H, Zouboulis C, Zhu M, Lee M. Dendrimer-conjugated isotretinoin for controlled transdermal drug delivery. J Nanobiotechnology. 2023;21(1):285.
38. Hu Q, Zhang F, Wei Y, Liu J, Nie Y, Xie J, et al. Drug-Embedded Nanovesicles Assembled from Peptide-Decorated Hyaluronic Acid for Rheumatoid Arthritis Synergistic Therapy. Biomacromolecules. 2023;24(8):3532-44.
39. Ren X, Ren J, Li Y, Yuan S, Wang G. Preparation of caffeic acid grafted chitosan self-assembled micelles to enhance oral bioavailability and antibacterial activity of quercetin. Front Vet Sci. 2023;10:1218025.
40. Huang YC, Zeng YJ, Lin YW, Tai HC, Don TM. In Situ Encapsulation of Camptothecin by Self-Assembly of Poly(acrylic acid)-b-Poly(N-Isopropylacrylamide) and Chitosan for Controlled Drug Delivery. Polymers. 2023;15(11):2463.
41. Mukhopadhyay P, Sarkar K, Chakraborty M, Bhattacharya S, Mishra R, Kundu P. Oral insulin delivery by self-assembled chitosan nanoparticles: In vitro and in vivo studies in diabetic animal model. Mater Sci Eng C. 2013;33(1):376-82.

42. Sezer AD, Sarılmışer HK, Rayaman E, Çevikbaş A, Öner ET, Akbuğa J. Development and characterization of vancomycin-loaded levan-based microparticular system for drug delivery. Pharm Dev Technol. 2017;22(5):627-34.
43. Hamley I. Lipopeptides: from self-assembly to bioactivity. Chem Commun. 2015;51:8574-83.
44. Aydin F, Chu X, Uppaladadium G, Devore D, Goyal R, Murthy NS, et al. Self-Assembly and Critical Aggregation Concentration Measurements of ABA Triblock Copolymers with Varying B Block Types: Model Development, Prediction, and Validation. J Phys Chem B. 2016;120(15):3666–76.
45. Yue Q, Luo Z, Li X, Fielding LA. 3D printable, thermo-responsive, self-healing, graphene oxide containing self-assembled hydrogels formed from block copolymer wormlike micelles. Soft Matter. 2023;19(34):6513-24.
46. Yuan Y, Wang Z, Su S, Lin C, Mi Y, Tan W, Guo Z. Self-assembled low molecular weight chitosan-based cationic micelle for improved water solubility, stability and sustained release of α-tocopherol. Food Chem. 2023;429:136886.
47. Sze-Tao K, Sathe S. Walnuts (Juglans regia L): proximate composition, protein solubility, protein amino acid composition and protein in vitro digestibility. J Sci Food Agric. 2000;80(9):1393-401.
48. Lv J, Zhou X, Wang W, Cheng Y, Wang F. Solubilization mechanism of self-assembled walnut protein nanoparticles and curcumin encapsulation. J Sci Food Agric. 2023;103(10):4908-18.
49. Greenfield, N. Using circular dichroism spectra to estimate protein secondary structure. Nat Protoc. 2006;1:2876–90.
50. Boott C, Gwyther J, Harniman R, Hayward D, Manners I. Scalable and uniform 1D nanoparticles by synchronous polymerization, crystallization and self-assembly. Nat Chem. 2017;9:785–92.
51. He Y, Grandi D, Chandradoss S, LuTheryn G, Cidonio G, Nunes Bastos R, et al. Rapid Production of Nanoscale Liposomes Using a 3D-Printed Reactor-In-A-Centrifuge: Formulation, Characterisation, and Super-Resolution Imaging. Micromachines. 2023;14(9):1763.
52. Bryant S, Elbourne A, Greaves T, Bryant G. Phytantriol phase behaviour in choline chloride urea and water mixtures. J Mater Chem B. 2023;11(29):6868-80.
53. Filipe V, Hawe A, Jiskoot W. Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the Measurement of Nanoparticles and Protein Aggregates. Pharm Res. 2010;27:796–810.
54. Sivakumaran M, Platt M. Tunable resistive pulse sensing: potential applications in nanomedicine. Nanomedicine. 2016;11(16):2197-214.
55. Jansook P, Pichayakorn W, Muankaew C, Loftsson T. Cyclodextrin–poloxamer aggregates as nanocarriers in eye drop formulations: dexamethasone and amphotericin B. Drug Dev Ind Pharm. 2016;42(9):1446-54.
56. Koch K, Dew B, Corcoran T, Przybycien T, Tilton R, Garoff S. Surface Tension Gradient Driven Spreading on Aqueous Mucin Solutions: A Possible Route to Enhanced Pulmonary Drug Delivery. Mol Pharmaceutics. 2011;8(2):387–94.
57. Alshamrani M, Ayon NJ, Alsalhi A, Akinjole O. Self-Assembled Nanomicellar Formulation of Docetaxel as a Potential Breast Cancer Chemotherapeutic System. Life. 2022;12(4):485.
58. Sun T, Han H, Hudalla G, Wen Y, Pompano R, Collier J. Thermal stability of self-assembled peptide vaccine materials. Acta Biomater. 2016;30:62-71.

59. Rarokar NR, Khedekar PB. Formulation and evaluation of docetaxel trihydrate loaded self-assembled nanocarriers for treatment of HER2 positive breast cancer. J Drug Deliv Ther. 2017;7(6):1-6.
60. ICH Q1A (R2) Stability testing of new drug substances and drug products - Scientific guideline [Internet] [cited 2023 Sep 10]. Available from: https://www.ema.europa.eu/en/ich-q1a-r2-stability-testing-new-drug-substances-drug-products-scientific-guideline.
61. Xie J, Wang C-H. Self-Assembled Biodegradable Nanoparticles Developed by Direct Dialysis for the Delivery of Paclitaxel. Pharm Res. 2005;22(12):2079-90.
62. Marino A, Battaglini M, Carmignani A, Pignatelli F, De Pasquale D, Tricinci O, Ciofani G. Magnetic self-assembly of 3D multicellular microscaffolds: A biomimetic brain tumor-on-a-chip for drug delivery and selectivity testing. APL Bioeng. 2023;7(3):036103.
63. Miller M, Chapa-Villarreal F, Oldenkamp H, Elder M, Venkataraman A, Peppas N. Stimuli-responsive self-assembled polymer nanoparticles for the oral delivery of antibodies. J Control Release. 2023;361:246-59.
64. Sarfraz M, Anjum F, Zahra D, Maqsood A, Ashfaq U. Recent Updates on Peptide Molecules in Drug and Vaccine Development. Curr Pharm Des. 2023. doi: 10.2174/1381612829666230717121632.
65. Chen YK, Simon IA, Maslov I, Oyarce-Pino IE, Kulkarni K, Hopper D, et al. A switch in N-terminal capping of β-peptides creates novel self-assembled nanoparticles. RSC Adv. 2023;13(42):29401-29407.
66. Hribernik N, Vargová D, Dal Colle MCS, Lim JH, Fittolani G, Yu Y, et al. Controlling the assembly of cellulose-based oligosaccharides through sequence modifications. Angew Chem Int Ed Engl. 2023:e202310357. doi: 10.1002/anie.202310357.
67. Dias AMGC, Moreira IP, Lychko I, Lopes Soares C, Nurrito A, Moura Barbosa AJ, et al. Hierarchical self-assembly of a reflectin-derived peptide. Front Chem. 2023;11:1267563.

Nosači lekovitih supstanci skloni samoorganizovanju: trenutni izazovi u karakterizaciji i izgledi za budućnost

Ivana Pantelić[1,*], Tanja Ilić[1], Ines Nikolić[1,2], Snežana Savić[1]

[1]Katedra za farmaceutsku tehnologiju i kozmetologiju, Univerzitet u Beogradu – Farmaceutski fakultet, Vojvode Stepe 450, 11221 Beograd, Srbija

[2]Section of Pharmaceutical Sciences, Institute of Pharmaceutical Sciences of Western Switzerland – ISPSO, Biopharmaceutical Sciences, University of Geneva, CMU – Rue Michel Servet 1, 1211 Geneva 4, Switzerland

*Autor za korespondenciju: Ivana Pantelić, e-mail: ivana.pantelic@pharmacy.bg.ac.rs

Kratak sadržaj

Pregled publikacija objavljenih poslednjih godina ukazuje na interesovanje za tzv. nosače sklone samoorganizovanju, kao i njihov potencijal za isporuku lekovitih supstanci različitim putevima primene. U ovom kontekstu, samoorganizacija označava proces relativno spontanog obrazovanja visoko uređenih agregata (koji ponekad zahteva specifične uslove – npr., pH, temperaturu, jonsku jačinu), zahvaljujući interakcijama različite prirode. Ovaj proces, karakterističan za mnoge supstance prirodnog porekla (određene polisaharide, proteine, lipide), poslužio je kao inspiracija istraživačima da osmisle i sintetišu inovativne materijale sklone samoorganizovanju, ili ispitaju kombinacije postojećih materijala. Ovaj rad pruža pregled najčešće ispitivanih materijala, odnosno nosača dobijenih samoorganizovanjem, koji često pripadaju sferi farmaceutske nanotehnologije. Nosači skloni samoorganizovanju mogu unaprediti stabilnost, efikasnost inkapsulacije i/ili kontrolisanu isporuku lekovitih supstanci. Ipak, raznolikost geometrija dobijenih nosača (sfere, poliedri, elipse, diskovi, porozne strukture, itd.) predstavlja značajan izazov za karakterizaciju, često zahtevajući primenu više komplementarnih tehnika, naročito za valjanu evaluaciju veličine i morfologije dobijenih nosača. Diskutovane su najčešće korišćene tehnike fizičko-hemijske i biofarmaceutske karakterizacije, uz isticanje njihovih prednosti i nedostataka. Na kraju, dat je kritički osvrt o izgledima za buduću primenu nosača lekovitih supstanci sklonih samoorganizovanju.

Ključne reči: materijali skloni samoorganizovanju, metode pripreme nosača, ispitivanje morfologije, tehnike procene veličine nosača, metoda sa dijaliznim vrećicama