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The Janus effect of colloidal self-assembly on the biological response of amphiphilic drugs

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ABSTRACT

In aqueous environment amphiphilic molecules organize themselves into supramolecular structures deeply affecting the chemo-physical properties. Supramolecular assemby is also crucial in the pharmaceutical development of bioactive lipophilic molecules whose attitude to self-aggregate is a recognized factor affecting the *in vivo* pharmacokinetic, but can also play a crucial role in the interaction with the biological targets in *in vitro* tests. In aqueous solution, amphiphilic drugs exist in a complex equilibrium involving free monomers, oligomers and larger supramolecular aggregates held together by noncovalent bonds. In this review we focus our attention on the dual effect of drugs self-assembly, which can both reduce the availability of active compounds and create multivalent scaffolds, potentially improving binding affinity and avidity to cellular targets. We examine the effect of aggregation on different classes of amphiphatic molecules with significant biological activities, such as immunomodulatory, anti-tumor, antiviral, and antibiotic.

Our purpose is to provide a comprehensive overview of how supramolecular chemistry influences the pharmacological and biological responses of amphiphilic molecules, emphasizing the need to consider these effects in early-stage drug development and *in vitro* testing. By elucidating these phenomena, this review aims to offer insights into optimizing drug design and formulation to overcome challenges posed by self-aggregation.

1. Introduction

The development of new pharmaceuticals is a complex and costly endeavor, often spanning many years and requiring substantial financial investment. This process is full of numerous unpredictable challenges that can hinder progress from initial discovery to market approval. Among the significant challenges in drug development, ensuring the bioavailability and efficacy of active pharmaceutical ingredients (APIs) is pivotal, particularly for compounds that are not readily soluble in aqueous environments, such as lipophilic and amphiphilic molecules [1].

Many of commercial drugs and pharmacologically active compounds, part of which are natural and/or nature-inspired, are amphiphilic substances able to self-aggregate in aqueous solutions [2].

Amphiphilic molecules, which possess both hydrophilic (water-attracting) and lipophilic (fat-attracting) components, are in fact a prominent class of compounds in the pharmaceutical industry. Because of their chemical structure, these molecules spontaneously arrange in aqueous solution into a large variety of morphologically different self-aggregate structures like micelles, vesicles, and bilayers, stabilized by non-covalent interactions and aimed to minimize the direct contact between hydrophobic part of molecules and polar environment [3,4], thus reducing the free energy of the system [5].

Micelles are spherical colloidal particles in water with hydrophobic tails directed inward and the hydrophilic heads face outward interacting with the aqueous environment. They are useful in drug formulations for

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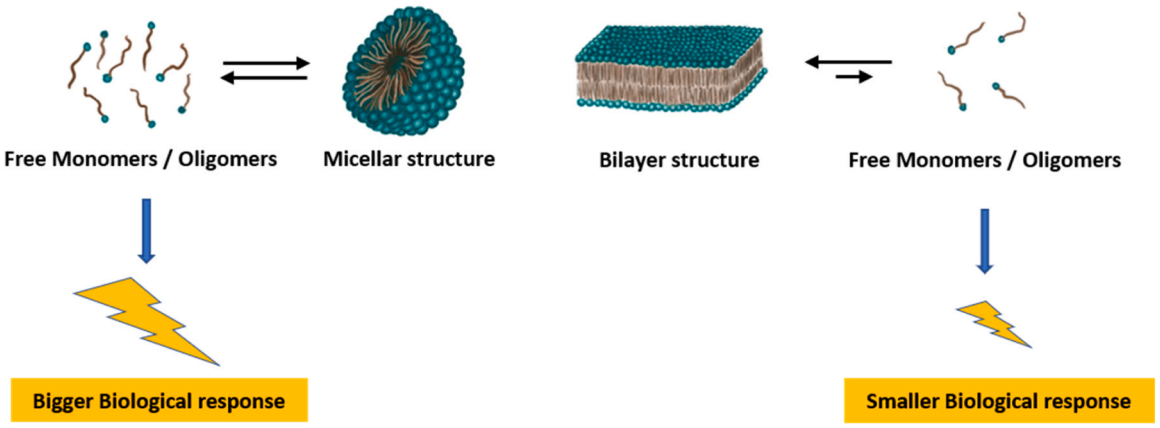

Fig. 1. Effect of the supramolecular aggregates on the pharmacodynamics.

enhancing the solubility of lipophilic drugs in aqueous solution and are characterized by a key parameter, the critical micelle concentration (CMC), indicating the concentration at which micelles begin to form.

Vesicles, based on bilayer organization, are the most widespread and important in nature, since they represent the structural unit of biological membranes. These supramolecular structures encapsulates an aqueous core, creating a compartmentalized unit. Vesicles are employed in drug delivery systems to enclose drugs and control their release over time ensuring a longer life.

Molecular structure [4,6], environmental conditions [7,8] and presence of other substances [9,10] represent crucial factors able to influence the morphology and stability of self-assembled structures. In this regard the number, length and composition of the hydrophobic tails as well as the nature of the hydrophilic head, affects the type and stability of the assemblies, as much as the temperature, pH, ionic strength and the addition of salts, surfactants, or other solutes could play crucial roles in the self-assembly process and behavior.

The ability of amphiphilic molecules to form organized structures can significantly influence their pharmacokinetic profiles, affecting absorption, distribution, metabolism, and excretion (ADME). However, while the pharmacokinetic implications of self-aggregation are well-recognized [11], its effects on *in vitro* assays are often underestimated. These tests, essential for early-stage drug discovery and develop, can be skewed by the presence of self-assembled aggregates, that could sequester the active drug, reducing its apparent concentration and availability for interaction with target molecules or cells, finally leading to an underestimation of potency and efficacy [11].

Additionally, the self-assembly-induced multivalency effect can enhance binding affinity to specific cellular receptors, possibly potentiating or inhibiting the desired biological response. [12] This effect can complicate the interpretation of *in vitro* data, as it may not accurately reflect the compound's behavior in a more complex *in vivo* environment. Understanding therefore this behavior could be crucial for accurate prediction of *in vivo* pharmacological responses and optimizing drug formulations.

This manuscript aims to explore how supramolecular chemistry influences the *in vitro* behavior of various amphiphilic bioactive molecules, including those with immunomodulatory, antitumor, antiviral, and antibiotic properties. We seek to illustrate how self-assembly can influence and modify pharmacological and biological responses, affecting the accuracy and reliability of screening tests. By providing a comprehensive overview of these phenomena, our purpose is to underscore the importance of considering supramolecular behavior in the early stages of drug development and to highlight how the self-assembly could mask and/or alter their pharmacological and biological response, affecting screening tests. This understanding is critical for accurately assessing drug candidates and developing effective therapeutic agents that can overcome the limitations imposed by self-aggregation.

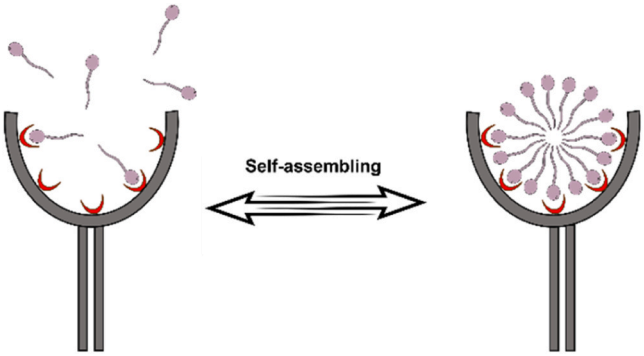

Fig. 2. Representation of multivalent interaction of self-assembled amphiphilic molecules.

2. Discussion

Spontaneous sopramolecular aggregration leads to assembly of amphipathic molecules held together by non-covalent binds [3] and stabilized by minimization of the contact between hydrophobic moieties and water. This chemo-physical process give rise to multiple equilibria involving free monomers, oligomers, and larger supramolecular aggregates such micelles, vesicles and lamellae (Fig. 1). Micellar structures were characterized by lower stability that allowed a dynamic and fast equilibrium with free monomers/oligomers. On the other hand, vesicles or lamellae are more stable and show slower rate of the equilibrium between assembly and disassembly, decreasing concentration of active monomers/oligomers [13–15], which is expected to reduce the availability of the active product to cell target, regardless of the biological and pharmacological intrinsic activity of the compound. Consequently, factors causing the shift of this equilibrium towards the less aggregated forms promote the availability of active molecules on target and consequently the biological response. Any element supporting the shift of aqueous equilibria towards more structured and stable supramolecular aggregates inhibited biological activity by hindering the presence of the necessary concentration of active forms, determining in this way a negative effect on the pharmacodynamics.

Supramolecular assembly can also affect positively the biological properties. In nature, multivalent aggregates are a widespread and frequent result of the interaction of molecules bearing more than one functional group. Scaffolds generated by these processes show binding properties different or with higher affinity in comparison to monomer-cell target recognition (Fig. 2) [12]. Naturally-occurring self-assembly

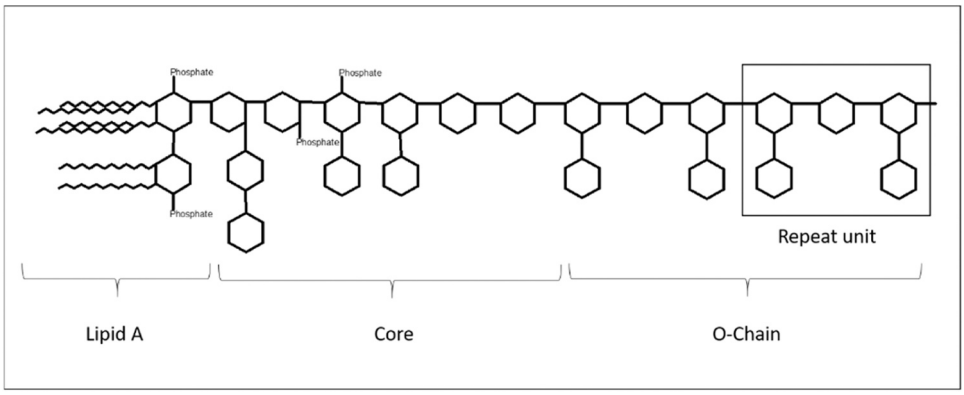

Fig. 3. LPS structure.

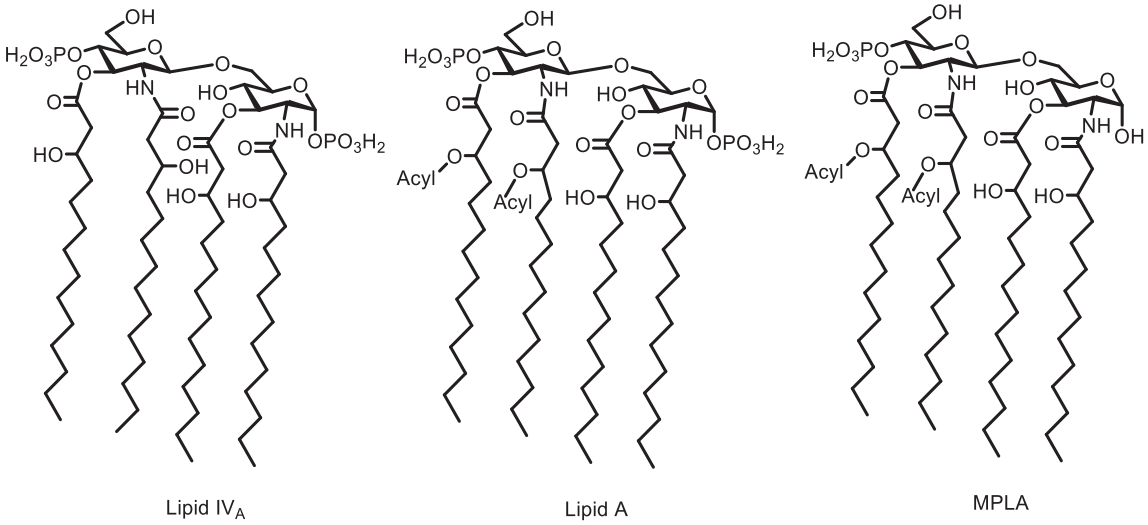

Fig. 4. Lipid IV_A, Lipid A and MPLA.

is a natural strategy to establish molecular interaction events, in particular on cell surface where receptors and/or ligands could assemble each other to determine a multivalent region [16]. Self-assembled nanostructures are also used to design multivalent scaffolding of multiple monomers and achieve synthetic nanosystems of biological interest [12,17–19].

2.1. *Negative effect of supramolecular aggregation on biological response*

2.1.1. *Immunostimulant LPS*

A first crucial case of negative effect of supramolecular aggregation on the biological activity, concerned the Lipopolisaccharide (LPS) (Fig. 3), main constituents of the outer membranes of gram-negative bacteria and responsible for the activation of the innate immune reaction by Toll-Like Receptor 4 (TLR-4)-dependent mechanism. They are carachterized by large molecular weight and chemical structure consisting of three regions: an outer polysaccharide region (O-Chain) including common hexoses, a short chain sugars part containing unusual sugars such as 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno heptose (Hep) along with common sugars (Core), and an inner region containing di-glucosamine unit with long-chain fatty acids and phosphate groups (Lipid A). The polysaccharide part is mainly responsible for serological specificity while the Lipid A moiety determines biological functions [20–22].

The immune response against LPS typically triggers release of proinflammatory mediators which, in moderate levels, could benefit the host by promoting inflammation and priming the immune system to eliminate possible invading pathogens. This response resulted strictly related to the nature, number and distribution of acyl chains and phosphate groups in the Lipid A moiety [22]. LPS forms aggregates in aqueous environments and the investigation of the relationship between biological activity and chemo-physical properties was potentially crucial to clarify the mechanisms underlying the immune response. The analysis of self-aggregates of LPS from *Escherichia coli* demonstrated the preferential formation of micellar structures with different size in physiological conditions [23–25]. Albeit the topic regarding the real active species, monomers or aggregates, was under discussion [26], the bioactivity concentrations of LPS, often lower than its critical micellar concentrations (CMC), would actually suggest that monomers were the main active forms, without excluding however the possible presence of metastable premicellar structures in equilibrium with active monomers. According to this, the evaluation of increased activity of monomeric endotoxin [27] or disaggregated LPS (above all monomers) [28,29] could confirm it. However, these studies were particularly hampered by the natural heterogeneity of the molecules.

Lipid IV_A [30–39] precursor of lipid A and monophosphoryl lipid A (MPLA) (Fig. 4) are purer and more homogeneous but less active amphiphilic glycolipids strictly related to LPS [36–39]. Studies of the chemo-physical behaviour of lipid IV_A highlithed the relevance of the self-assembly investigation in the interpretation of biological response to LPS [30,31].

Analysis of quasi-elastic light scattering (QELS) and small angle X-

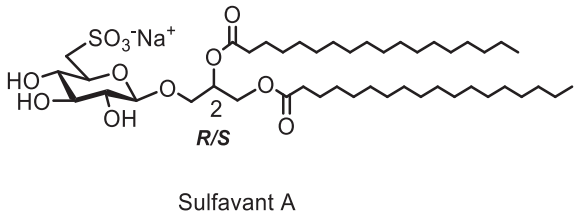

Fig. 5. Sulfavant A.

ray (SAXS) showed the formation in water of large unilamellar vesicles (250 nm), in agreement with minor extension of the polar portion compared to more active LPS, usually in micellar form [23–25,32–35]. However, the data also supported a possible co-occurrence of vesicles and micelles [30,31].

Furthermore considering the correlation between the biological activity of lipid A from different bacterial sources and the ability to assume particular supramolecular structures, it was evident that lamellar-arranged lipid A were inactive. Coversely lipid A, in mixed lamellar/micellar cubic forms, presented intermediate activity until to highly active analogues structured in non-lamellar organization [40], suggesting the relevance of chemo-physical state in water as a crucial factor for biological outcome.

2.1.2. *Immunomodulatory sulfavants*

Recently we characterized a new molecular immunomodulator, named Sulfavant A (Fig. 5), able to elicit an unprecedented immune response by the engagement of the triggering receptor expressed on myeloid cells-2 (TREM2) [41–48], and under preclinical phase as novel vaccine adjuvant. Sulfavant A ([1,2-*O*-distearoyl-3-*O*-(β-sulfoquinovosyl)-*R/S*-glycerol]) is a synthetic β-sulfoquinovosyl-distearoyl glycerol inspired to the carbon skeleton of natural α-sulfoquinovosyl-diacylglycerols (α-SQDG). The molecule induced maturation of human Dendritic Cells (DCs) at micromolar concentrations following a bell-shaped dose-response curve, typical of several amphiphilic molecules [16,47,49].

In vivo experiments showed that the sulfolipid was able both to boost immune protection in mice and to inhibit tumour growth in a model of cancer vaccine against melanoma [41]. From a chemical point of view, Sulfavant A is a mixture of *R/S* epimers at the carbon 2 of the glycerol moiety (Fig. 6). The two epimers Sulfavant S and Sulfavant R were separately synthetized and immunomodulatory assays showed a surprising activity of DCs maturation at nanomolar concentrations, 1000-fold lower their mixture [41–43,46–48], with a bell-shaped dose-response curve (Fig. 7).

Chemical reactivity and biological activity of sulfoquinovosides are deeply affected by the colloidal properties in water [50–53], but investigation of supramolecular assembly in physiological environment is often hampered by simultaneous occurrence of several aggregation forms and low analysis concentrations.

Sensitive cryogenic transmission electron microscopy (Cryo-TEM) allowed to bypass these troubles, permitting the study of supramolecular organization of Sulfavants across the entire activity concentration range (micromolar-nanomolar) [54]. Analysis of these sulfolipids showed formation of colloidal particles with different size and stability, proving a dissimilar supramolecular organizative behaviour between the pure epimers, Sulfavant R and Sulfavant S, and the epimeric mixture Sulfavant A [46,47,54].

In particular at nanomolar concentrations, micelles were the predominant self-assembled forms for the more biologically active epimers, while for their mixture the occurring small and more stable vesicles determined absence of biological activity. As concentrations increased to micromolar values, Sulfavants were capable of spontaneously forming vesicles with divergent size and stability. The epimers self-assembled in smaller and more stable aggregates, showing a loss of biological activity at these concentrations, differently from the Sulfavant A less stable ones, therefore active at micromolar concentrations (Fig. 8).

Considering the minor stability of micellar structures [54], we suggested that at nanomolar concentration the amount of the free monomers for Sulfavant R and Sulfavant S could be higher than that of Sulfavant A. A reverse situation occurred at micromolar concentrations. As conclusive evidence, addition of detergents to water suspensions of Sulfavant A induced a significant increase of the potency from micromolar to nanomolar activity range, highlighting the negative impact of the self-aggregation for the biological response once again. Considerations on this study led to conclude that the different aggregation behaviours of Sulfavants modified the biological activity, affecting the effective concentration of the free monomers at cellular target, determining a variation of immunological efficiency.

In addition to substances whose activity was linked to the recognition of membrane receptors, there are many others lipophilic drugs exerting their pharmacological actions inside the cells and whose chemo-physical behavior impacted in the same way on biological response.

2.1.3. *DNA intercalating agents Antracyclines*

Anthracyclines (Fig. 9) are class of antibiotics used in cancer chemotherapy for their ability to bind both DNA and DNA-binding enzyme topoisomerase-II [55]. These molecules are intercalating agents of DNA, thus inhibiting both DNA replication and RNA transcription. 2D-Nuclear Overhauser Enhancement (NOESY) spectra, Diffusion Order Spectroscopy (DOSY), absorption and fluorescence

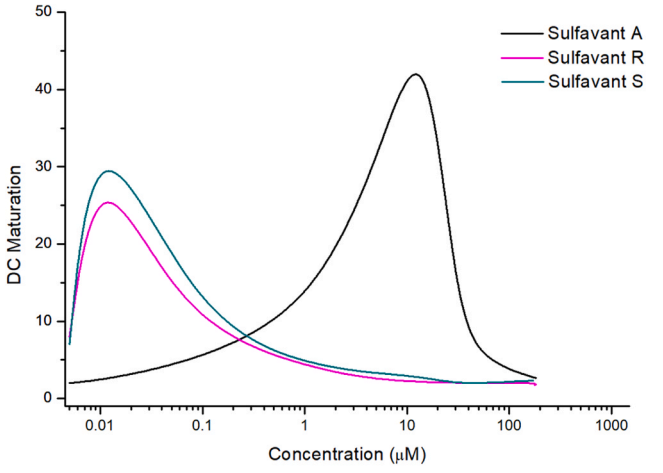

Fig. 7. DCs maturation expressed as percentage of $CD83^{+}$ cells depending on Sulfavants concentration [54], [55].

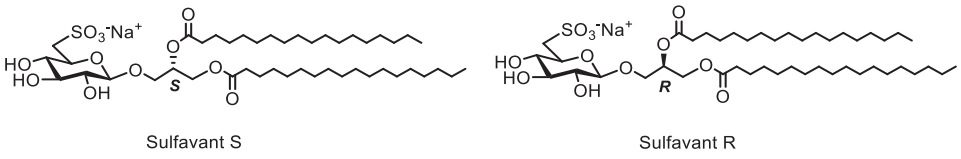

Fig. 6. Sulfavant S and Sulfavant R.

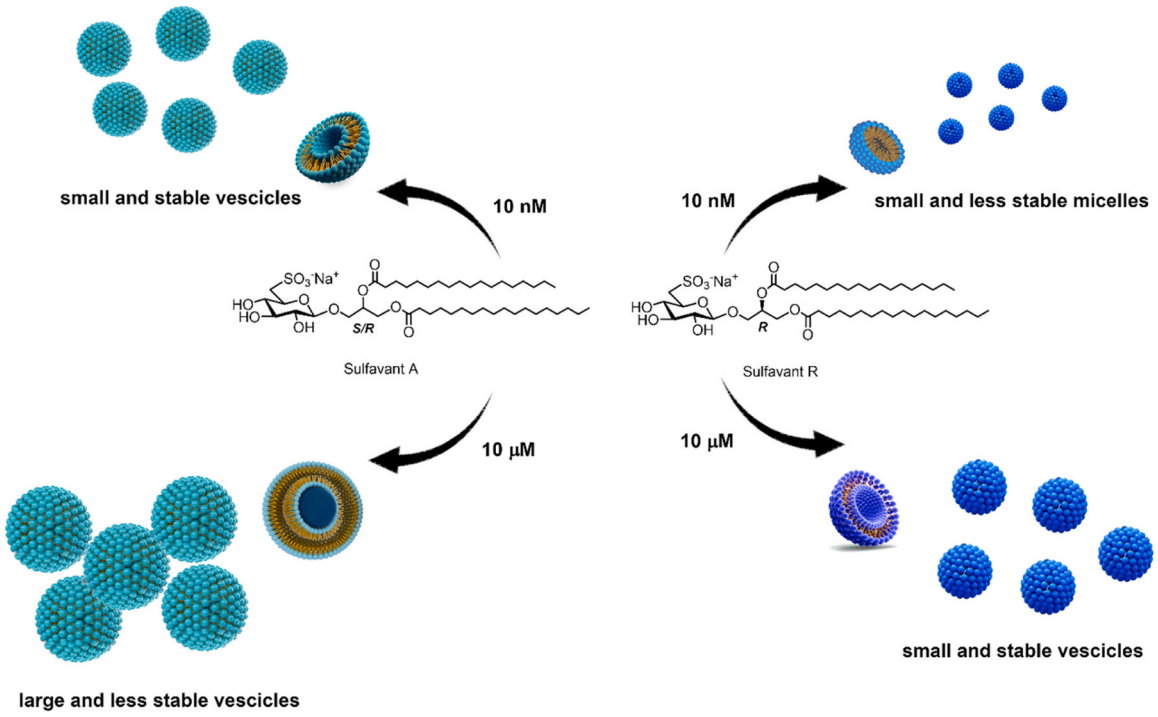

Fig. 8. Representation of self-assembling of aqueous solution of Sulfavant A and the *R* epimer at 10 nM and 10 μM concentration [54].

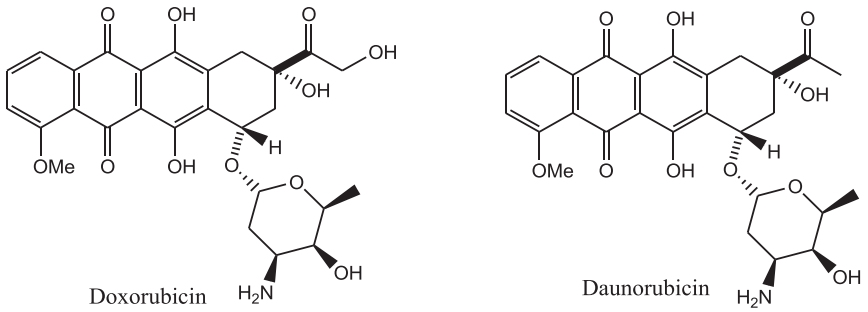

Fig. 9. Anthracyclines derivatives.

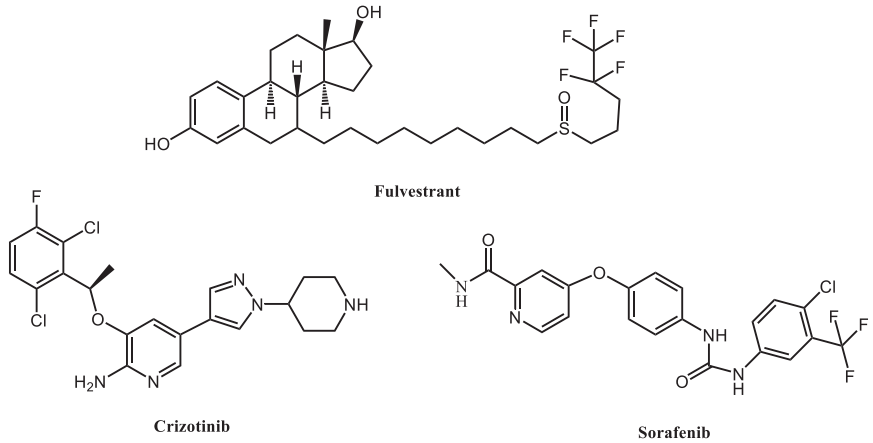

Fig. 10. Fulvestrant, Sorafenib, and Crizotinib.

studies and Electron Spray Ionization Mass Spectrometry (ESI-MS) analysis demonstrated that in aqueous solution, the anthracyclines were capable to form self-assembled structures driven by π-stacking interaction of aromatic moieties, that compete with the DNA binding for the monomers sequestration. The complexity of the aggregates is concentration-dependent and dimers are the predominant form at low

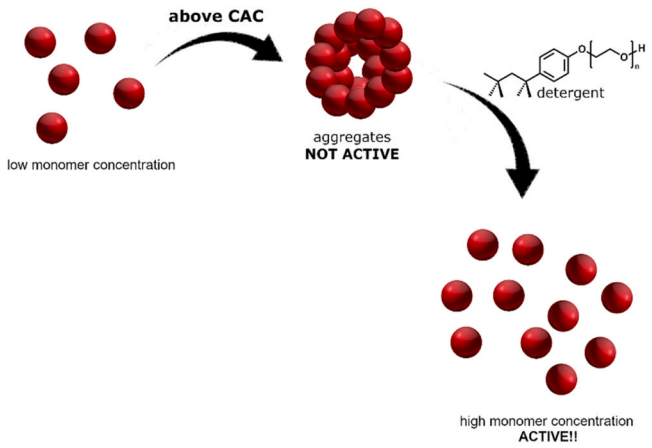

Fig. 11. Effect of the addition of nonionic detergents on Anticancer Drugs.

concentration. The stability and cohesive strength among bioactive monomers in supramolecular structure deeply influenced the monomeric availability to the cell nucleus target, influencing in this way their anticancer action [56].

2.1.4. *Anticancer drugs fulvestrant, sorafenib and crizotinib*

Negative influence of supramolecular aggregation on biological response and bell-shaped dose-response curve for lipophilic drugs has been discussed by Owen et al. [57]. According to these authors, colloidal structures in cell environment are a form of storage of monomers which reduces the concentration and biological effectiveness of the molecules. The anticancer drugs Fulvestrant, Sorafenib and Crizotinib (Fig. 10) lose their pharmacological activity above their micromolar critical aggregation concentrations for the formation of colloidal aggregates of 82, 69 and 163 nm in size respectively (measured by DLS). The activity trend was comparable to the stability of supramolecular aggregation forms. In fact, the biological effect was significantly favored using surfactants, as Tween-80, that consequently also increased the toxic effect (Fig. 11).

2.2. *Positive effect of supramolecular aggregation on biological response*

2.2.1. *Aggregation-induced multivalency effect*

Multivalence is a spontaneous phenomenon that makes many amphiphilic molecules capable of binding their cellular targets more avidly, determining a positive effect on the biological response. In this regard, lipid-bearing carbohydrates represent an indicative example. A multivalent aggregate was reported by self-assembly of sialic acid derivatives (Fig. 12) [58]. The supramolecular structure recognizes and inhibit the viral adhesion protein hemagglutinin with greater affinity than single monomers, thus determining a more effective antiviral effect respect to monovalent sialosides.

Lipid-bearing sialic acid as inhibitors of viral attachment to cell membranes have been also reported by Sun et al. [59]. Analogously, different mannose-bearing amphiphiles form vesicles or spherical and worm-like micelles that inhibit the concavanalin A-induced erythrocyte agglutination with hundreds-fold greater efficiency compared to the single mannose (Fig. 13) [60–62]. These multivalent aggregates could

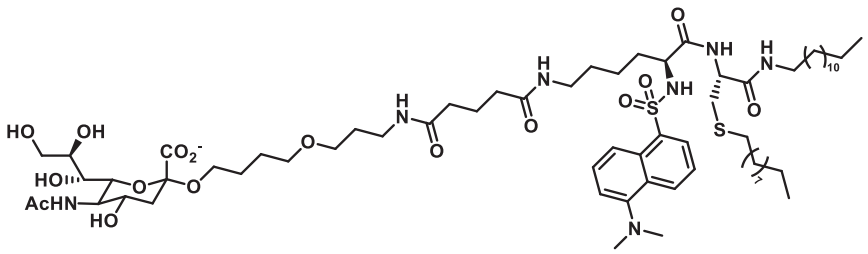

Fig. 12. Chemical structure of synthetic amphiphilic derivative of sialic acid.

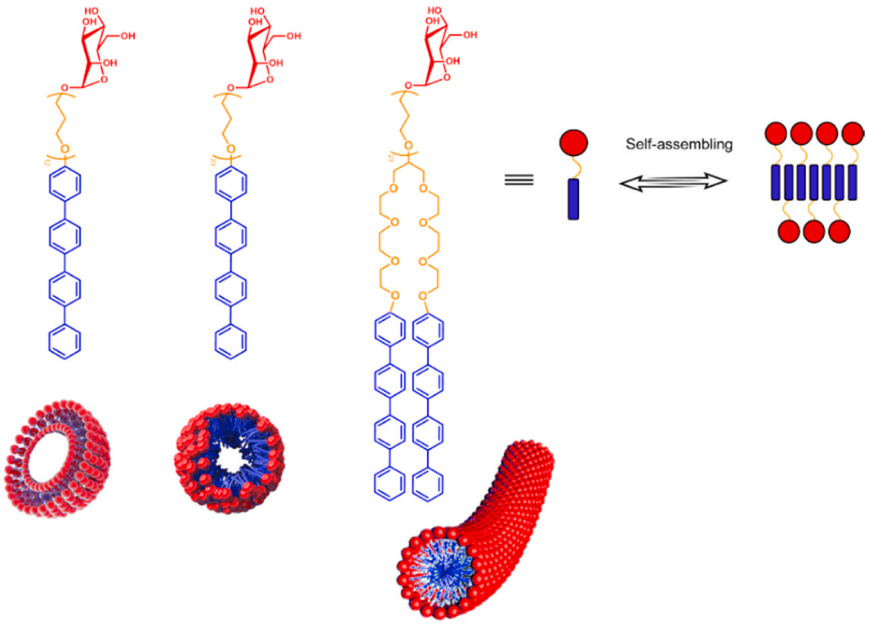

Fig. 13. Schematic representation of mannose-functionalized aggregates [61].

also bind to *E.coli* bacteria surface with affinity higher than the single momomers. The size and form of the final aggregates affect the bioactivity significantly, with the result that micellar forms were most potent inhibitors, probably due to the higher surface curve [61].

Considering the spontaneity of the self-assembly process, this behavior can be used to simulate or hinder many biological processes. In particular, given the large structure of the assembled products, this approach could be widely employed to bind systems like proteins, nucleic acids, viruses, and bacterial and human cells.

3. Conclusions

In aqueous solution, self-assembly is a spontaneous process involving the organization of amphipathic molecules in supramolecular structures. Many of commercial drugs and pharmacologically active compounds are lipophilic substances able to self-aggregate in physiological conditions. This behaviour has not always been fully evaluated in relation to its influence on the pharmacological response as well as its correlation with pharmacodynamic and pharmacokinetic aspects that are crucial for pharmacological development. The aqueous self-assembly induce complex equilibria involving both free monomers and larger supramolecular aggregates. The poor assessment of these effects of the spontaneous process is due to the analytica difficulty in physiological environment at the low concentrations where they take place. Nonetheless, this phenomenon often affects the interactions of lipophilic drugs with the biological targets and leads to alteration in the pharmacological activity.

The chemo-physical behaviour in water of LPS, sulfolipids, anthracyclines and the anticancer drugs fulvestrant, sorafenib and crizotinib, support the assumption that self-assembly greatly affects the amount of the active monomer available to interact with the biological target. As proved with these molecules, the active concentration depends on the type, dimension and the stability of occurring supramolecular structures which in turn proved to be strongly dependent on the range of concentrations considered. Opposite effects of self-assembly were highlighted by natural or induced colloidal organization of lipidated sialic- and mannose-based carbohydrates, able to determine more efficient multivalent interactions with cellular target, consequently increasing the biological response.

Therefore self-aggregation seems producing negative effect when the activity is dependent on specifc ligand-target binding due to the reduction of the effective concentration of the single monomer but it becomes positive when are involved multivariate interactions, such as multiligand entities, because of the increase of avidity and stability of the active complex.

Finally, in *in vivo* and *in vitro* results with bioactive amphiphilic substances are consequence of the natural aptitude of these molecules to self-aggregate in aqueous solution. Pharmacological investigation of these molecules should not ignore the attention on their supramolecular aggregates properties in order to avoid underestimation and misinterpretation of the biological activity of these compounds.

CRediT authorship contribution statement

Marcello Mercogliano: Writing – review & editing, Data curation. **Giuliana d'Ippolito:** Writing – review & editing. **Daniela Castiglia:** Software, Formal analysis. **Carmela Gallo:** Writing – review & editing. **Genoveffa Nuzzo:** Writing – review & editing. **emiliano manzo:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation, Conceptualization. **Angelo Fontana:** Writing – review & editing, Writing – original draft, Conceptualization. **Laura Fioretto:** Writing – review & editing, Data curation. **Marcello Ziaco:** Writing – review & editing, Data curation.

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

Data will be made available on request.

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Competing financial interest

The authors declare no competing financial interests.

Ethical Issue

The authors declare no ethical issue.

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