ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

REVIEW ARTICLE

Cyclodextrin-Based Nanosponges -A Promising Nano technological Drug Delivery System

Hassanien Sagban Taghi*, **Haider J. Al-Karagully**

Department of Pharmaceutics, College of Pharmacy, Al-Bayan University/Iraq.

***Corresponding Author: Hassanien Sagban Taghi**

Abstract

Undoubtedly, Cyclodextrin-based nanosponges (CD-NSs) are patentable nanosized, efficient delivery system designated as a three-dimensional nanostructure network. It can form porous spherical nanoparticles with a crystalline or amorphous structure. The internal polarizability of NS can be easily adjusting by alternating the type of cross-linker (CL) molecules and degree of cross-linking. Cyclodextrin-based nanosponges are a safe and can form complexes with different types of lipophilic or hydrophilic molecules. The nanosponges could be used to improve the aqueous solubility of poorly water-soluble molecules, protect degradable substances, obtain sustained delivery systems or design innovative drug carriers for nanomedicine.

Keywords: *Cyclodextrin, Nanosponges, Drug Delivery System.*

Introduction

Nanotechnology could simply define as the technology at the scale of one-billionth of a meter. It is the use on a miniature scale. It is the layout, synthesis, and application by monitoring shape and size at nanometer scale [1].

Nanotechnology in Drug Delivery Systems

Nanotechnology is the creation and utilization of materials, devices, and systems through the control of matter on the nanometer- length scale. It is the familiar term for designing and utilization of structures with at least one characteristic dimension measured in nanometer [2].

The inclination toward miniaturization of carriers had already started prior to the introduction of nanotechnology in drug delivery. The rightness of nanoparticles for use in drug delivery depends on a variety of individualities, including size and porosity [3].

Due to the smaller size of nanostructures, drugs can dissolve more rapidly and can pass safely through the body's smallest blood vessels then, infiltrate into targeted tissues [40. Panyam and Labhasetwar stated that the uptake of nanoparticle through tissues was found to be 15-250 times greater than that of microparticles in the 1- 10µm range [5].In addition to these obstacles, more than half of active substances identified through combinatorial screening programs are difficult to formulate for administration due to their significant insolubility in water [6, 7].

In order to alleviate these challenges of conventional drug delivery, significant interest has been focused on the development of delivery systems that can enhance therapeutic solubility, release the drug in a sustained manner, preferentially localize the therapeutic to the site of action, and overall enhance therapeutic efficacy [8]. During the past decade, some of nano-formulations have been clinically approved or are under clinical investigation (Table 1) [9, 10].

Table 1: Some Nano- formulations of drugs that are approved for Clinical Use or under Clinical Trials

Nanonization strategy	Trade name	Drug	Indication	Dosage form	Developer, status
High-pressure homogenization	Triglide®	Fenofibrate	Hypercholesterolemia	Oral tablet	SkyePharma/Sciele, approved in 2005
Media milling	Rapamune®	Sirolimus	Immunosuppression	Oral tablet	Elan/Wyeth, approved in 2000
	Megace®	Megestrol	Antianorexia,	Oral suspension	Elan/Par,Pharmaceuticals approved in 2005
Nanoemulsion	Estrasorb®	Estradiol	Vasomotor symptoms	Topical emulsion	Novavax/Graceway, approved in 2003
	Restasis®	Cyclosporine	Chronic dry eye disease	Ophthalmic emulsion	Allergan, approved in 2002
Polymeric micelles	Genexol-PM	Paclitaxel	Solid tumors	Lyophilized powders for suspension	Approved in South Korea in 2007, Phase II in the US
	NK911®	Doxorubicin	Solid tumors	Lyophilized powders for suspension	Nippon Kayaku, Phase II

Recently, nanoparticles delivery systems have received plenty of concern which assigned to be a versatile drug delivery system [11]. So, the Figure (1) displays a different delivery system in term of nanoparticles.

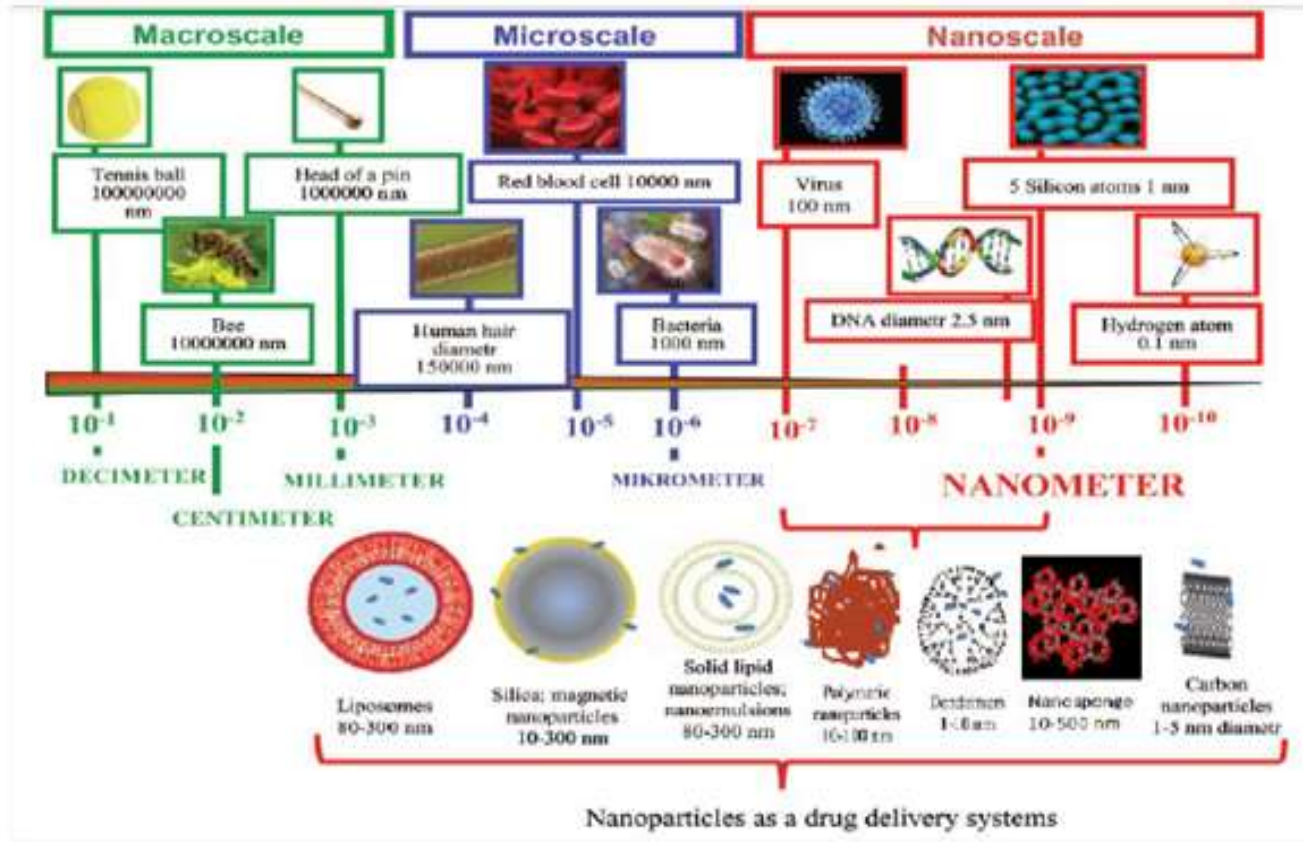

Figure 1: Nanoparticle drug delivery systems with relation to other scales

Classifications of Nanoparticles

Nanoparticles substantially divided into the following groups [12].

- ✓ Lipid-based nanoparticles.
- ✓ Lipid–polymer hybrid nanoparticles.
- ✓ Polymer-based nanoparticles.

Polymer-Based Nanoparticles

These nanoparticles are nano-sized colloidal particles in which drug could be instilled or encapsulated within their matrix or adsorbed or conjugated onto the surface [13].

According to the method of linking with drugs, the polymeric nanoparticles are of three types:

- Complexing nanoparticles: when nanoparticles attached to the drug molecules by electro-static charges.
- Conjugating nanoparticles: at which the drug covalently linked to the nanoparticles.
- Encapsulating nanoparticles: These are stated by nanocapsules and nanosponges (NS). Nanosponges are sponge like nanoparticles containing many holes that carry the drug molecules. In contrast, nanocapsules type, in which the drug is pent to a cavity of CD-NS at the nanoscale level [14].

Nanosponges

Nanosponges thought of being a unique category of polymer-based nanoparticles, delineated to be a hyper-crosslinked structure with mixture sizes and nanosized cavities typically obtained by natural derivatives [15].

Types of Nanosponges

Nanosponges made of many different organic or inorganic materials. Accordingly, could be; titanium-based nanosponges [16], silicon nanosponge particles [17], metal ion nanosponges [18], hyper cross-linked polystyrene nanosponges [19], carbon-coated metallic nanosponge [20], and of course, cyclodextrin-based nanosponges [21]. Well - known examples amongst the varied varieties of nanosponges, CD-NS have gained significant attention and widely studied.

Cyclodextrin-Based Nanosponges

The hunt for an efficient drug delivery system is an enigma in itself. Our medical researchers and formulation scientists are always surrounded by issues pertaining to drug targeting, drug release, overdosing, solubility, permeability, activity, and bioavailability. Thus, creating or improving systems for drug delivery is a vicinity of current analysis [22].As soon as nanotechnology has turned up in the limelight, its bright prospects in medicine have been hailed enthusiastically. The advent of cyclodextrin-based nanosponges has paved the way to alleviate the above limitations and has gained a tremendous impetus in drug delivery [10].

The terminology 'cyclodextrin nanosponges' (Figure 2) was originally used by De Quan Li and Min Ma in 1998 to signify a ß-cyclodextrin, cross-linked by diisocyanates which when observed microscopically showed a porous structure and had a very high inclusion constant with quite a lot of organic pollutants [23].

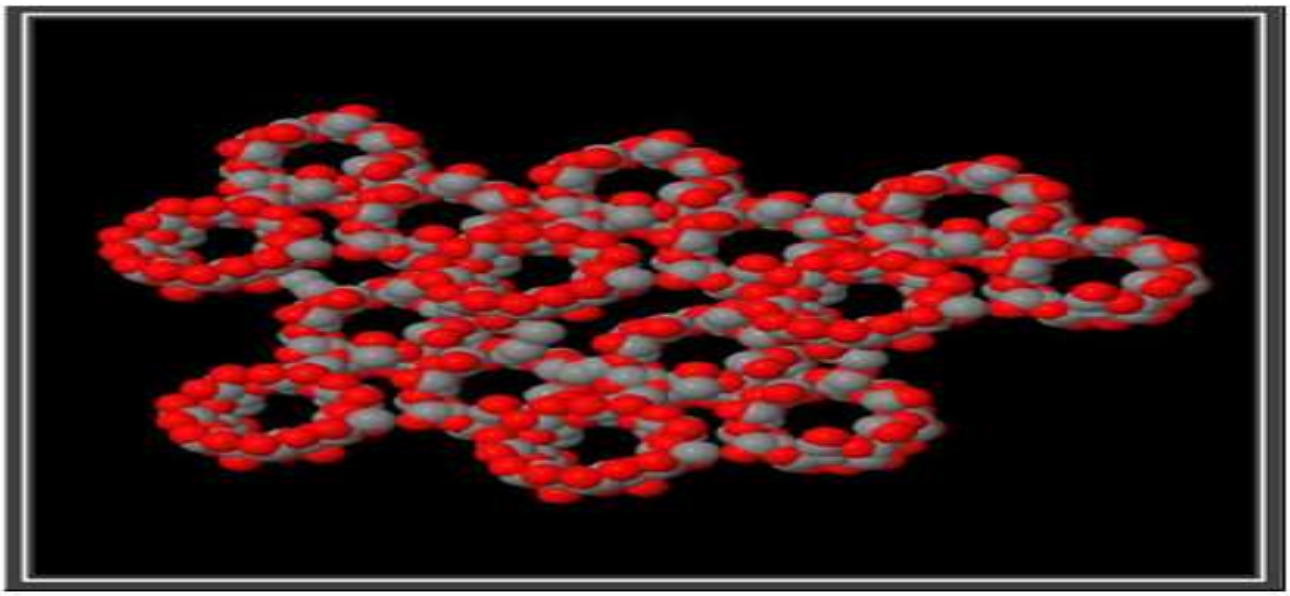

Figure 2: Molecular structure of cyclodextrin nanosponges

Cyclodextrin-based nanosponges are the lyophilized sponge-like structures (Fig. 3); appear as a three-dimensional network or scaffold, tiny, mesh-like structures. Internally, they comprise of a numerous of intersecting cavities within a non-collapsible structure with tunable polarity and cavities of nanometric size, microscopically, less than 1 micrometer diameter, that can be loaded with a wide variety of drugs, and morphologically, they are seemed to be spherical with the typical porous exterior surface [24]. These hyper-cross-linked cyclodextrins can be obtained by reaction of

hydroxyl groups of α, ß and γ cyclodextrins, with appropriate polyfunctional small molecules called cross-linker (CL) to form solid reticulate with hydrophobic sinus where materials can be entrapped [25].These carriers have been candidate for drug, with considerable and high solubilizing efficiency for poorly soluble drugs through forming inclusion and non-inclusion complexes with various drugs. Moreover, they improve the bioavailability of medications [26].

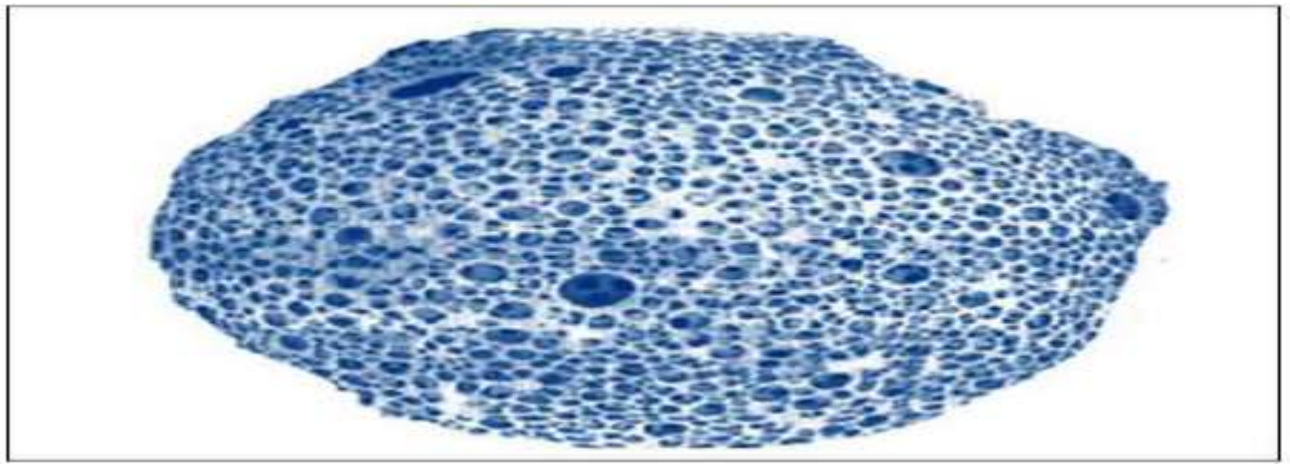

Figure 3: Conceptual structure of cyclodextrin nanosponges

Interesting Features of Cyclodextrin-Based Nanosponges

The NSs exhibit a range of (1 µm or less) with the tunable polarity of their cavities [27].The drug release from these NS can be customized by tuning the type and degree of crosslinking. As they are biocompatible, nontoxic, safe, free-irritating, non-mutagenic, free-allergenic, and solid in nature, they could be designed as different dosage forms like oral, inhalational and parenteral dosage forms [28, 29].

They possessed a desirable zeta potential, which makes them, sufficiently high to produce stable suspension. as well, they are stable enough at robust temperatures up to 300°C and over the pH range of 1 to 11 [25]. Different drugs could be included within nanosponge by inclusion and non-inclusion complex [30].Another important feature is that the formation constant depends closely on the solvent used. In aqueous environments, inclusion formation is greatly favored. On the contrary; the inclusion process is completely reversible in organic and less polar solvents, such as ethanol. Because of this reversibility, nanosponges are easily regenerated simply by washing with an ecofriendly solvent such as ethanol [31].

Advantages of Cyclodextrin-Based Nanosponges

Nanosponges possess unique benefits that make them different from other particulate systems as listed in the following [32, 33]:

- They possess numerous advantages ranging from improved solubility, stability, permeation, bioavailability, modulation of drug release to efficient drug targeting. Also, delivery of proteins, enzymes and gases are feasible.
- By nanosponges, liquids may be converted to powders and unpleasant flavors can be masked. Thus, the improvement in formulation flexibility and processing of materials could be achieved
- They are self-sterilizing, which make an organism difficult to penetrate NS pore.
- They are cost effective.

Disadvantage of Cyclodextrin-Based Nanosponges

The fundamental hindrance of NSs is their capability to incorporate solely little molecules. Also, their loading capability counts chiefly on the degree of crystallization [34].They might be either para-crystalline or in crystalline kind, reckoning on the method conditions. Para-crystalline NS has shown numerous drug loading capacities, on the opposite hand, next kind of NS plays an important role in their complexation with medication [26, 35].

Formation of Inclusion Complex

A well-known feature of the CDs is its known ability to form solid inclusion complexes, these complexes are dimensional fitting between host cavity and guest molecule (Fig. 4) at the lipophilic cavity of CD provides a

good media into favorable sized non-polar moieties can enter to form inclusion complexes [36].

Indeed, no covalent bonds are broken or fashioned throughout formation of the complex, however the most propulsion of comples formation is that the unharness of enthalpy-rich water molecules from the cavity associate degreed displaced by a lot of hydrophobic guest molecules within the answer to realize an apolar-apolar link and reduce of CD ring strain leading to a more stable, lower energy conjugated by van der Waal forces [37].

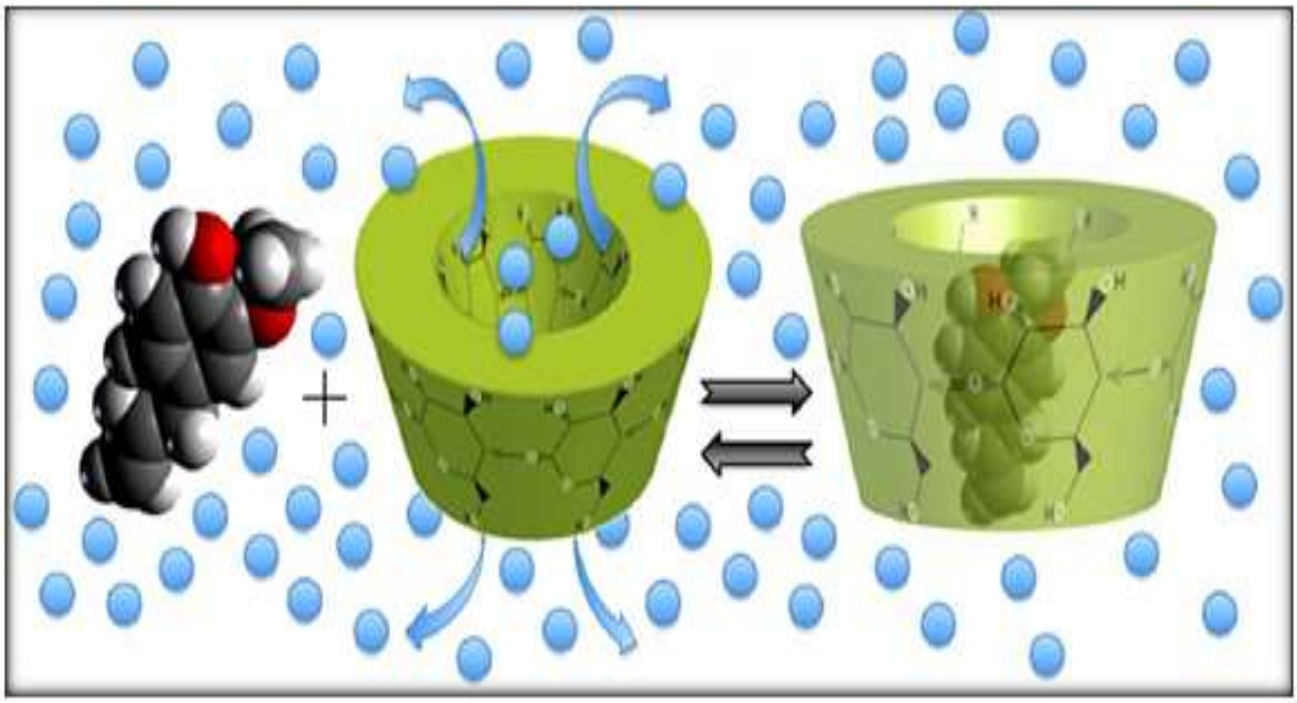

Figure 4: Inclusion complex formation (host-guest complex)

Methods of Preparation of Cyclodextrin-based Nanosponge

Indeed, two approaches used for the preparation of NS are condensation polymerization and interfacial polymerization.

Condensation Polymerization

As the name 'condensation' suggests, cyclodextrins have cross-linked the presence of an excess quantity of CL preferably the ones with the active carbonyl containing the substance, for example carbonyl diimidazole, triphosgene, diphenyl carbonate, and organic dianhydride. [34].Once the reaction gets over, the mixture is allowed to cool and is poured into an excess of distilled water. Vacuum filtration recovers the product.

Prolonged soxhlet extraction process obtains the purified product by suitable solvent (ethanol or acetone) which is followed by grinding in the mill to get the powder form of the cyclodextrin-based nanosponges [42]. Condensation polymerization technique could be simply achieved by one of following two common methods in below:

Solvent Method

These include mixing with the CDs in a polar aprotic solvent for example anhydrous dimethylformamide; anhydrous dimethyl sulfoxide after that, adds resulting solution to an excess of CL, the molar ratio of (CD: cross linker) is preferred as 1:2 to 1:12. The reaction proceeds with a solvent reflux temperature higher than 130-140°C and time ranging from 1 to 48 hr [36].

The reaction is finished as a clear reticulate block fashioned, that has proved after that, the mixture allowed to cool to ambient temperature, then is added to an excess of double distilled water, and product is recovered by filtration under vacuum and at the same purify by prolonged soxhlet extraction with ethanol or acetone. Eventually, the product is dried under vacuum and grinded in a mechanical mill to get homogeneous powder [37]. The steps of nanosponge preparation by the solvent method are represented in Figure (5).

Ultrasound-assisted Melting Method

NSs are resulted through reacting CD with CLs without using solvent and sonification is

maintained. The size of yielded NS mainly will be spherical and uniform [26]. Schematic diagram of ultrasound-assisted synthesis steps of nanosponge is represented in Figure (6).

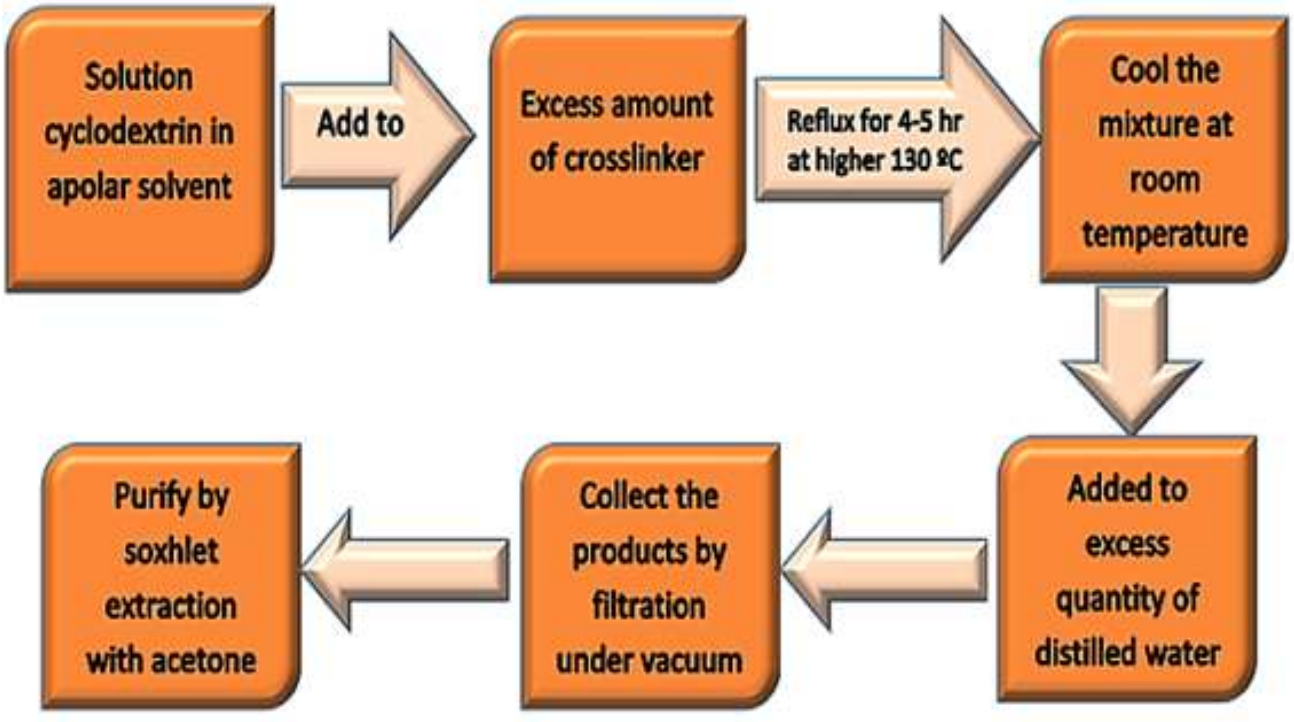

Figure 5: Steps involved in the preparation of cyclodextrin-based nanosponges by solvent technique

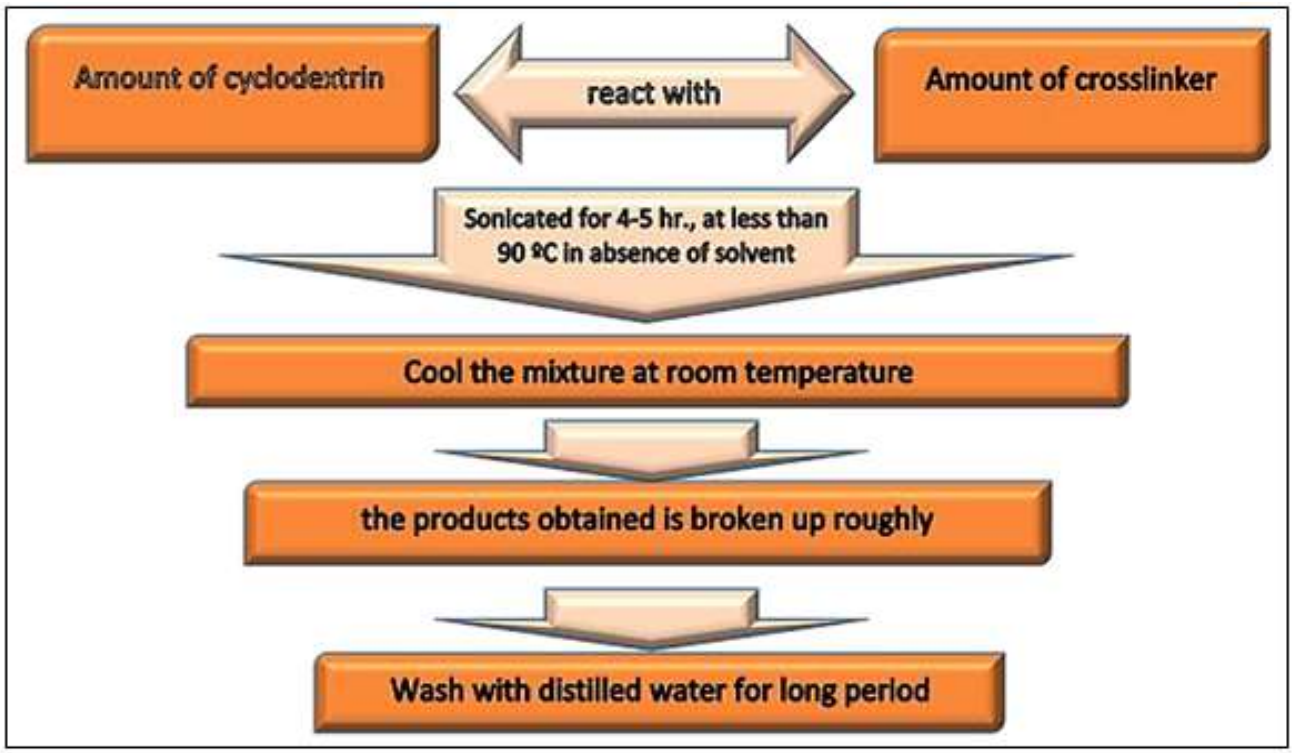

Figure 6: Steps involved in the preparation of cyclodextrin based nanosponges by ultrasound-assisted synthesis [38]

Interfacial Polymerization

It is a method for preparing cyclodextrin nanosponges (CDNS) including the steps (illustrated in Figure 7) of dissolving CD in an alkali aqueous solution (potassium hydroxide) with a pH more than 10 to form a CD solution, dissolving cross-linking agent in organic solvent (methylene chloride, butanone, and chloroform) to form a solution, and then, solutions were added together with continuous agitation for half hour [32]. The yield was rinsed with purified water and centrifuged at 3000 rpm for 15 minutes. The filtrate was dried in vacuum conditions to obtain the nanosponge [39].

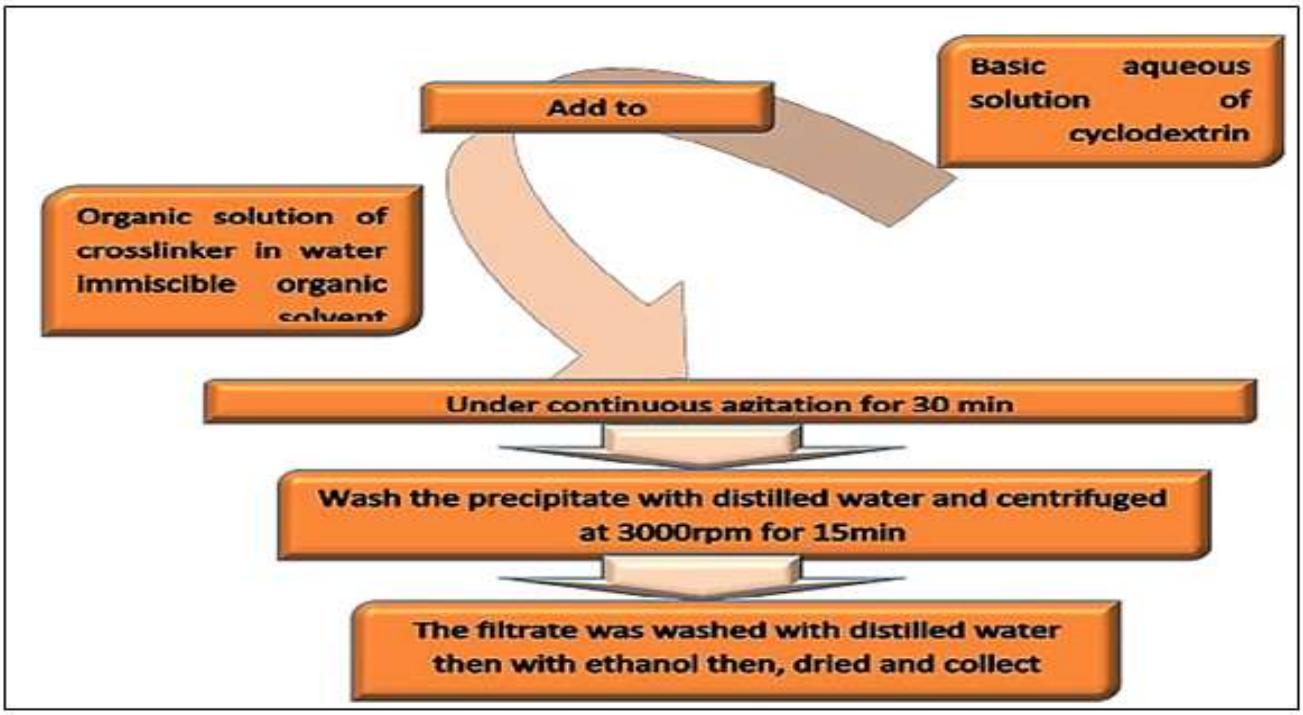

Figure 7: Steps involved in the preparation of cyclodextrin-based nanosponges by interfacial polymerization [38]

Characterization of Nanosponges

A comprehensive characterization of cyclodextrin-based nanosponges, including Fourier transform infrared radiation (FTIR), Raman spectroscopy, X-ray diffraction, microscopic studies, thermal analyses, solubility studies, zeta potential and the like help in confirming the inclusion complexes formed [24].

Application of Cyclodextrin-based Nanosponges

These nanosponges hold a tremendous promise in enhancing various attributes that pose a problem for formulation scientists including concerns pertaining to solubility, stability, bioavailability, activity, permeation and the like, which makes a formulation challenging [26].

Solubility Enhancement

Undoubtedly, NS has been utilized to sufficiently enhance the solubility of the drug in addition to their distinctive role in improving the dissolution rate of poorly soluble drugs as scientifically proven as well as modifying release profile in a well-controlled manner, throughout formations of inclusion complex suitable drugs [40].

Modulation of Drug Release

The release of the included drugs within cyclodextrin-based nanosponges can varied according to the type of nanosponges and crosslinking degree. So, release kinetics may be modified and accelerated [41, 42].

Stability Enhancement

Cyclodextrin-based nanosponges can retard degradation of encapsulated molecules from light, enzymatically or chemical without itself getting involved in the reaction [43].

Reduction in Volatility of Essential Oils

Cyclodextrin based nanosponges is possible to induce protection of volatile components like (Linalool) against loss by evaporation, thereby, in perfumes. Hence, we can have a long-lasting effect by slow release of the chief volatile components [44].

Nanosponges as Carriers for Biocatalysts, Enzymes, Proteins, Vaccines and Antibodies

It is worth mentioning that protein, peptide, and enzymes are used in the medical purposes. Still, there have been numerous potential hurdles in its therapy like denaturation, aggregation, and adsorption such as short half-life, immunogenicity, rapid enzymatic degradation [45].

Nanosponges as Carrier for Delivery of Gases

The inclusion of gases in CD cavities has already been proven, thus has now been made possible. Trotta F et al. prepared ß-CD nanosponges and they approved that NS been able to store large amounts of CO_2 [46].

Cavalli et al. developed oxygen as cyclodextrin-based nanosponges to delivered oxygen topically to heal wounds [47].

Conclusion

Nanosponges area unit a brand new sort of biocompatible cross-linked CD, whose production is versatile and efficient. CD-NS possess explicit feature concerning their encapsulation ability, and descriptive solubility enhancing with respect to differing kinds of molecules. Furthermore, NSs might broaden the vary of applications of CD in pharmacy, in addition as in different important fields, like agriculture, and cosmetics. NS Possesses an impressive ability to delivery two active drugs, simultaneously for medical aid purposes and concurrent therapeutic needs.

Lastly, nanosponges will Encouraging the further application of novel nanotechnology be thought-about as multifunctional nanoscale systems appropriate for the delivery of active drugs in nanomedicine.

References

1. Pandey P1, Purohit D2, Dureja H1 (2018) Nanosponges -A Promising Novel Drug Delivery System; Recent Pat Nanotechnol., 12(3):180-191.
2. Miyazaki K, Islam N (2007) Nanotechnology systems of innovation-An analysis of industry and academia research activities. Technovation, 27: 661-671.
3. Kocbek, P, Baumgartner S, Kristl J (2006) Preparation and evaluation of nanosuspensions for enhancing the dissolution of poorly soluble drugs. International Journal of Pharmaceutics, 312 (1-2): 179-186.
4. Kewal K Jain (2014) Drug Delivery System. Second Edition. Springer Science+ Business Media.
5. Panyam J, Labhasetwar V (2003) Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Delivery Rev, 55(3): 329-347.
6. Van Vlerken L, Amiji E (2006) Multi-functional polymeric nanoparticles for tumour-targeted drug delivery. Expert Opinion on Drug Delivery, 3 (2): 205-216.
7. Merisko L, EM Liversidge GG (2008) Drug nanoparticles: formulating poorly water-soluble compounds. Toxicologic Pathology, 36(1): 43-48.
8. Lipinski CA (2001) Drug-like properties and the causes of poor solubility and poor permeability. Journal of Pharmacological and Toxicological Methods, 44 (1): 235-249
9. Marcato PD, Duran N (2007) New aspects of nanopharmaceutical delivery systems. J. Nanosci. Nanotechnol., 8: 2216-2229.
10. Huabing C, Chalermchai K (2011) Nanonization strategies for poorly water-soluble drugs. Drug Discovery Today, 16: 7-8.
11. Zhang C, Awasthi N, Schwarz M A, Hinz S, Schwarz RE (2013) Superior antitumor activity of nanoparticle albumin-bound paclitaxel in experimental gastric cancer, PLoS One, 8: 58037.
12. Nimesh S, Manchanda R, Kumar R, Saxena A, Chaudhary P, Yadav V, Mozumdar S, Chandra R (2006) Preparation, characterization and in vitro drug release studies of novel polymeric nanoparticles. Int. J. Pharm., 323: 146-152. 57.
13. Labhasetwar V, Song C, Levy RJ (1997) Nanoparticle drug delivery system forrestenosis. Advanced Drug Delivery Reviews, 24(1): 63-85.
14. Maria D, Moya O, Carmen AL (2012) Cyclodextrin-based nanogels for pharmaceutical and biomedical applications. International Journal of Pharmaceutics, 428: 152-163.
15. Guo L, Gao G, X Liu, F Liu (2008) Preparation and characterization of TiO2 nanosponge, Mater. Chem. Phys., 111: 322-325.
16. Zuruzi A.S, Kolmakov A, MacDonald NC, Moskovits M (2006) Highly sensitive gas sensor based on integrated titania nanosponge arrays. Appl. Phys. Lett., 88: 102-113.
17. Farrell D, Limaye S, Subramanian D (2006) Silicon Nanosponge Particles, U.S. Pat 0,251,561A1, 9.
18. Abu Samah Z (2007) Metal oxide “nanosponges” as chemical sensors: Highly sensitive detection of hydrogen using nanosponge titania, nanostructural material; Angewandte Chemie-international Edition, 46 (23): 4298-4301.
19. Dakankov V, Llyin M, Tsyurupa M, Timofeeva G, Dubronina L (1998) From a

dissolved polystyrene coil to intramolecularly hyper cross linked nanosponges, Macromolecules, 29: 8398-8403.

20. Lian K, Wu Q (2000) Carbon encased metal nanoparticles and sponges, methods of synthesis and methods of use. IPC patent AB01D5394FI.
21. Swaminathan S, Pastero L, Serpe L, Trotta F, Vavia P, Aquilano D, Trotta M, Zara G, Cavalli R (2010) Cyclodextrin-based nanosponges encapsulating camptothecin: Physicochemical characterization, stability and cytotoxicity, Eur. J. Pharm. Biopharm., 74: 193-201.
22. Malve NV, Gadhave MV, Banerjee SK, Gaikwad DD (2014) Nanosponge: A Novel Approach in Drug Delivery System. International Journal of Institutional Pharmacy and Life Sciences, 4(2):16-26.
23. Li D, Ma M (2000) Nanosponges for water purification. Clean Technol. Environ. Policy, 2(2):112-16.
24. Ewelina W, Fabrizio Caldera, Trotta F (2014) Peroxidase-encapsulated cyclodextrin nanosponge immunoconjugates as a signal enhancement tool in optical and electrochemical assays. Analyst, 139: 375.
25. Indira B, Bolisetti S, Samrat Ch, et al (2012) Nanosponges: a new era in drug delivery:review. J Pharmacy Research, 5(12):5293-6
26. Trotta F, Cavalli F, Tumiatti V, Zerbinati O, Roggero C, Vallero R (2007) Ultrasound Assisted Synthesis of Cyclodextrin Based Nanosponges, EP Pat 1786841A1.
27. Trotta F, Marco Z, Roberta C (2012) Cyclodextrin-based nanosponges as drug carrier. Beilstein J. Org. Chem., 8: 2091-2099.
28. Patel E, Oswal R (2012) Nanosponge and microsponges: a novel drug delivery system, Int. J. Res. Pharm. Chem., 2: 237-244.
29. Nilesh J, Ruchi J, Navneet T, Brham Prakash G, Deepak Kumar J (2010) Nanotechnology : A Safe and Effective Drug Delivery Systems, Asian Journal of Pharmaceutical and Clinical Research, 3(3): 159-165.
30. Mamba BB, Krause RW, Malefetse TJ, Sithhole SP (2008) Cyclodextrin nanosponges in the removal of organic matter to produce water for power generation. Water SA, 34: 657-660.
31. Trotta F, Shende P, Blasizzo M (2012) Method for preparing dextrin nanosponges. WO 2012/147069 A1, 1.
32. Trotta F, Cavalli R (2009) Characterization and application of new hyper-cross-linked cyclodextrins, Compos. Interfaces, 16: 39-48.
33. Trotta F, Cavalli R, Tumiatti W, Zerbinati O, Roggero C, Vallero R (2006) Ultrasound assisted synthesis of cyclodextrin based nanosponges. WO 2006/002814 A1.
34. Cavalli R, Trotta F (2006) Cyclodextrin-based Nanosponges for Drug Delivery. Journal of Inclusion Phenomena and Macrocyclic Chemistry, 56: 209-213.
35. Schneiderman E, Stalcup AM (2000) Cyclodextrins: A Versatile Tool in Separation Science. J. Chromatogr B, 745: 83-102.
36. Lala R, Thorat A, Gargote C (2011) Current trends in ß-cyclodextrin based drug delivery systems, Int. J. Res Ayur. Pharm., 2(5): 1520-1526.
37. Sai VC, Priti PP (2014) Cyclodextrin-based nanosponges: a propitious platform for enhancing drug delivery. Expert Opin. Drug Deliv., 11(1):111-120.
38. Ashwini D, Pritesh P. Preparation and Evaluation of Cyclodextrin based Atorvastatin Nanosponges. Am. J. PharmTech Res. 2014; 4(3).
39. Hassanien Sagban Taghi, Alaa A Abdul Rasool, Yehia I Khalil (2016) Enhancement of tenoxicam solubility by HP-cyclodextrin based nanosponge; World Journal of Pharmacy and Pharmaceutical Sciences, 5: 4.
40. Singireddy A, Subramanian S (2014) Fabrication of cyclodextrin nanosponges for quercetin delivery: physicochemical characterization, photo stability, and antioxidant effects. J Mater Sci., 49: 8140-8153.
41. Trotta F, Tumiatti V, Vallero R (2004) Nanosponges based on cyclodextrins functionalized with carboxyl groups: synthesis and use in the decontamination from heavy metals and organic compounds, Cyclodextrin News, 3rd European Conference on Cyclodextrin, 27: 11.
42. Liang W, Yang C, Nishijima M, et al (2012) Cyclodextrin nanosponge-sensitized enantiodifferentiating photoisomerization of cyclooctene and 1, 3-cyclooctadiene. Beilstein J. Org. Chem., 8: 1305-11.

43. Buschmann HJ, Schollmeyer E (2002) Applications of cyclodextrins in cosmetic products: A review. J. Cosmet. Sci., 53:185-91.
44. Ranucci E, Ferruti P, Manfredi A, Cavalli R (2012) Cross-linked Resins by Stepwise Polyaddition of ß-Cyclodextrin with Bisacrylamides and Assessment of Their Potential as pH-Sensitive NPs for Site-Specific Protein Delivery. In Proceedings CRS 2012, Quebec City, Canada, 15-17 99.
45. Barbara B, Trotta F (2010) High catalytic performances of Pseudomonas fluorescens lipase adsorbed on a new type of cyclodextrin-based nanosponges. Journal of Molecular Catalysis B: Enzymatic, 62: 155-161.
46. Gilardi G, Trotta F, Cavalli R, Ferruti P, Ranucci E, Di Nardo, Roggero C, Tumiatti V (2009) Cyclodextrin Nanosponges as Carrier for Biocatalysts, and in the Delivery and Release of Enzymes, Proteins, Vaccines and Antibodies, WO2009149883 A1, 17.

