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AQUASOMES: A SELF ASSEMBLED NANOCARRIER APPROACH FOR NOVEL DRUG DELIVERY OF BIOPHARMACEUTICALS

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ABSTRACT

Aquasomes are nanoparticles that are the most recently created drug delivery system and are ideal for the delivery of protein and peptides to overcome their limitations of chemical or physical instability, poor bioavailability typically caused by gastrointestinal enzymatic degradation and also the poor permeability of intestinal mucosa, through the oral route of administration. Aquasomes are novel rounded nanoparticulate drug carrier systems that vary in size from 60 to 300nm. They have a tri-layered structure with a solid core that provides structural stability for proteins and peptides, allowing for enough time for drug distribution in systemic circulation. These properties made the aquasomes as a prospective carrier system for the delivery of peptide based biopharmaceuticals and also designed for delivery of nanoparticulates like Anti-diabetic polypeptides such as- insulin and polypeptide-K, hemoglobin and various antigens, enzymes like serration peptidase via oral route.

KEYWORDS

Aquasomes, Nanocrystalline, Ceramic Nanoparticles, Hydroxyapatite core, Non-Covalently, Oligosaccharides and Peptides.

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INTRODUCTON

The aquasomes are one of the emerging approach and ideal choice of drug delivery comprises of the nano-particulate self-assembled carrier system[1]. Aquasomes are nano-particulate carrier system, these are three-layered self-assembled structures, comprised of a solid phase Nanocrystalline core coated with an oligomeric film to which biochemically active molecules are adsorbed with or without modification[2]. Alternatively, aquasomes are termed as "Bodies of Water", their water like properties support and sustain fragile biological molecules such as polypeptide and proteins. The function of preserving confirmative integrity and a

high degree of surface exposure is used to target and deliver bioactive molecules such as peptides and protein hormones, antigens and genes to specific locations where the action is required[3]. The aquasomes made up of ceramic core are stabilized by carbohydrates and by using methods like co-polymerization, diffusion or adsorption; the pharmacologically active molecules are incorporated on to the carbohydrate surfaces of preformed nanoparticles[4]. Aquasomes composed of three layered structure: Solid crystalline core, carbohydrate coat and the active drug which are self-assembled by non-covalent bonds. The core is coated with polyhydroxyoligomers onto which bioactive molecules are adsorbed. The solid core provides structural ability while carbohydrate coating plays an important role and acts like a natural stabilizer which protects against dehydration and stabilizes the biochemically active molecules[5]. Aquasomes are spherical 60-300nm particles. Aquasomes offer an attractive mode of delivery for drugs which are having problems such as route of delivery, physical as well as chemical instability, poor bioavailability and potent side effects[6]. Formulations of aquasomes are mainly administered by parenteral route but new studies suggest that it could also be administered by other routes. Aquasomes delivers their bioactive molecules via a combination of special targeting molecular shielding and sustained release process[1].

Nanocarriers increase the therapeutic efficacy of the pharmaceutically active agents as they can regulate their release, improve their stability and prolong circulation time by protecting the drug from phagocytosis and premature degradation. These delivery vehicles have the potential to augment the pharmacodynamic and pharmacokinetic profiles of drug molecules, thereby enhancing the therapeutic efficacy of the pharmaceutical agents[7]. Targeting of the drug to specific site is always a challenging task. So the scientists came across some novel drug delivery systems which mainly include vesicular, colloidal, niosomal, microparticulate, nanoparticulate, and lipid-based submicron system[8,9]. Nanoparticles are versatile nanocarriers as these can be fabricated from the polymer or ceramics. Polymeric nanoparticles can be made from biological such as albumin/gelatin or from organics such as acrylates. Likewise ceramic nanoparticles can be fabricated from crystalline carbon and calcium phosphate core. Ceramic nanoparticles are ceramic based, spherical, nano-size carriers that consist of a hydroxyapatite core whose surface is non-covalently modified by oligosaccharide on which bioactive material/ drug are further adsorbed. These carbohydrate stabilized ceramic nanoparticles are also known as ‘’aquasomes”. The particle size of aquasomes is lower than 1000nm which is suitable for parenteral administration[10]. Some researchers have extended the research about the route of administration for aquasomes from parenteral to oral, e.g., Srivani prepared the sugar coated ceramic nanocarriers of hydrophobic drug (piroxicam) for oral delivery[11]. Aquasomes possess properties of maintaining conformational integrity, and a high degree of surface exposure, which is successfully targeted for the delivery of peptide molecules such as insulin, hemoglobin; enzymes like serratiopeptidase[1] and also the conformational integrity of aquasomes exploited as a red blood cell substitutes, vaccines for delivery of viral antigen (Epstein-Barr and Immune deficiency virus) to evoke correct antibody and as targeted system for intracellular gene therapy. The delivery system has been successfully utilized for the delivery of insulin, hemoglobin, and various antigens. Oral delivery of enzymes like serratiopeptidase has also been achieved. So, Aquasomes are one of the most recently developed delivery systems that are finding a niche as peptide and protein carriers[12,13].

Administration of proteins and peptides in their active state has been a formidable challenge to the pharmaceutical as well as biotechnological industries. Drug associated challenges such as suitable route of drug delivery, physical and chemical instability, poor bioavailability, and potentially serious side effects of these bioengineered molecules are some potential limitations on their successful formulation. The combination of biotechnology and nanotechnology (i.e., nanobiotechnology) has proposed a new

approach as a solution to their formulation problem in the form of aquasomes[14,15]. Aquasomes are able to overcome those inherent problems. The surface modification with carbohydrates creates a glassy molecular stabilization film that adsorbs therapeutic proteins with minimal structural denaturation. Thus, these particles provide complete protection of an aqueous nature to the adsorbed drugs against the denaturing effects of external pH and temperature, because there are no swelling and porosity changes with change in pH or temperature[16].

PROPERTIES OF AQUASOMES

Aquasomes prevent clearance via the reticulo-endothelial system or degradation by other environmental problems because of their size and structural stability.

Due to its large size and active surface, Aquasomes can be loaded with adequate amounts of agents via non-covalent, ionic bonds, Vander Waals and entropic forces successfully[17].

The mechanism of action of aquasomes is governed by their surface chemistry.

The combination of various processes like specific targeting, molecular shielding, and slow and sustained release processes involved in the delivery of the active drug via aquasomes[18].

Aquasomes as a carrier also protects the drug/antigen/ protein from harsh pH conditions and enzymatic degradation, thus requiring lower doses[19].

Objectives of aquasomes

The main objective of preparing aquasomes is to protect bio-actives.

Aquasomes maintain molecular conformation and optimum pharmacological activity[20].

Many other delivery systems like pro-drugs, liposomes are prone to destructive interactions between drug and carrier while aquasomes have carbohydrate coating prevents destructive denaturing interaction between drug and solid carriers[21].

Aquasomes with natural stabilizers like various poly-hydroxy sugars act as dehydro-protectant, help in maintaining water-like state and preserves molecules in dry solid-state, protecting from the change in aqueous state, pH, temperature, solvent, salt causing denaturation[18,22].

An active molecule has characteristics such as unique three-dimensional conformation, freedom of internal molecular rearrangement caused by the freedom of bulk motion molecular interactions, but protein initiates irreversible denaturation when desiccated, even unstable in an aqueous state[23,22].

PRINCIPLE OF SELF-ASSEMBLY

Self-assembly means that in two or three dimensional space, the constituent parts of any final product assume the spontaneously prescribed structural orientations. The three-layered structure is self-assembled by non-covalent bonds[17]. Self-assembly in the aqueous environment of macromolecules have the virtue to design itself in smart nano-structured materials, which is primarily governed by three physicochemical processes;

Interaction between charged groups

Such as amino, carboxyl, sulfate, and phosphate groups, facilitate the long-range approach of self-assembling subunits. From the biological environment, the intrinsic chemical groups or adsorbed ions impart a polarity charge to biological and synthetic surfaces. Most biochemically related molecules are amphoteric molecules[20]. For the first phase of self-assembly, the long-range interaction of constituent subunits begins at an intermolecular distance of around 15nm. Long-range forces can extend to 25nm in the case of hydrophobic structures. Charged groups also play a part in the stabilization of folded protein tertiary structures.

Hydrogen bonding and dehydration effect

Hydrogen bond plays an important role in base pair matching and help to stabilize the secondary protein structure such as alpha helices and beta sheets. Hydrophilic molecules that form hydrogen bonds, provides the surrounding water molecules with a significant degree of organization. Whereas hydrophobic molecules unable to form hydrogen bonds, having the ability to repel water molecules from their surroundings to organize the moiety. Organized water decreases the entropy of the surrounding environment. Since it is

thermodynamically unfavorable, the molecule dehydrates and get self-assembled[23].

Structural stability

In the biological environment, structural stability of protein determined by the interaction between charged group and hydrogen bonds largely external to the molecule and by Vander Waals forces which responsible for hardness and softness of molecule and maintenance of internal secondary structures, provides sufficient softness, allows maintenance of conformation during self-assembly[20].

Vander Waals forces often experienced by the relatively hydrophobic molecular regions that are shielded from water play a subtle but critical role in maintaining molecular conformation during self-assembly[24]. In the case of aquasomes, sugars help in molecular plasticization[25].

COMPOSITION OF AQUASOMES

Aquasomes are composed of 3 main layers as shown in Figure No.1

Core material

Widely used core materials are ceramic and polymers. Ceramics such as diamond particles, brushier (calcium phosphate) and tin oxide are crystalline easy to manufacture, biodegradable in nature, low cost and biocompatible. It provides a high degree of order and structural regularity. Due to the high degree of order, higher surface energy yields which leads to efficacious binding of carbohydrate onto it. These properties make it a good candidate for aquasome formulation. (18) Polymers such as albumin, gelatin or acrylate are used[26].

Coating material

Cellobiose, pyridoxal 5 phosphate, sucrose, trehalose, chitosan, citrate etc. are widely used coating materials. As a natural stabilizer, carbohydrate plays a crucial role and it is preferred mostly. Carbohydrates are adsorbed as a glassy film in nanometer size range coating the preformed ceramicnanoparticles and self-assembled calcium phosphate dihydrate particles (colloidal precipitation)[9].

The carbohydrate used this purpose are as:

Cellobiose

It is 4-O-beta-D-glucopyranosyl-D-glucopyranose reducing sugar. It is acquired from the partial hydrolysis of cellulose. It protects the drug molecule against the dehydration[27].

Trehalose

It is an alpha-D-glucopuyranosyl-alpha D-glucopyranoside non-reducing sugar. Trehalose also shields the drug molecule against dehydration and denaturation. It is observed more effective than cellobiose[27]. It shown to be tolerant of stress in fungi, bacteria, insects, yeast and some plants. Trehalose acts by protecting proteins and membranes within plant cell during the desiccation process and thus preserves cell structures, inherent flavors, colors and texture[28].

Disaccharides like sucrose, trehalose contain a large number of hydroxyl groups and help to replace the water with polar protein residues. Thus it maintains their integrity in the absence of water. Experimental studies done with Calcium transporting microsomes isolated from rabbit muscles and lobster muscles showed that the structure and function of cellular components could be protected by sugar during lyophilization[29]. The rehydrated vesicles displays drastically decreased calcium-uptake and uncoupled activity of ATPase when Calcium transporting microsomes are lyophilized without stabilizer sugar. Lyophilized vesicles are morphologically distinct from newly prepared vesicles in presence of as little as 0.3g of trehalose per g. membrane upon rehydration[28,30].

Bio-active molecules

Drugs which have the property of interacting with the film via non-covalent and ionic interactions proved a good candidate for aquasomes[29].

Among three layers of aquasomes, carbohydrate fulfills the objective of aquasomes. The hydroxyl groups on carbohydrate interact with polar and charged groups of proteins, in the same way as with water thus preserve the aqueous structure of proteins on dehydration[30].

STRATEGIES USED IN THE CHEMICAL SYNTHESIS OF AQUASOMES

The strategies involved in chemical synthesis of these nanostructures are:

Sequential covalent synthesis

This strategy is used to produce arrays of covalently linked atoms with well-defined composition, connectivity and shape such as Vitamin B12, it can produce the structures that are distant from the thermodynamic minimum for collection of atoms[30].

Covalent polymerization

This strategy is used to prepare molecules with high molecular weight. Low weight substances are permitted to react with it to yield molecules, including many covalently associated monomers[32]. Such as Formation of polyethene from ethylene. Covalent polymerization indirectly provides synthetic routes to stable nanostructures and phase-separated polymers[4].

Self-organizing synthesis

This strategy depends on weaker and less directional bonds such as hydrogen, ionic and Van der Waals interactions to assemble atoms, ions or molecules into structures[18]. The different types of formulation are prepared by the use of this strategy include molecular crystals, ligand crystals, emulsions, colloids, micelles, self-assembled monolayers and phase-separated polymers. The ion or molecules adjust their position to reach the thermodynamic minimum and get self-organize, during formulation[33].

Molecular self-assembly

Self-assembly is a process in which a disorganized structure with pre-existing components forms an organized structure or design. Aquasomes self-assembly has various interesting applications in nanoscience and nanotechnology formulation development[31,32].

METHOD OF PREPARATION OF AQUASOMES

Aquasomes preparation is very simple and effortless process which requires minimum solvent usage and no homogenization steps. By using the principle of self-assembly[5], it involves three steps- flow Chart No.1.

Ceramic core is prepared first by a different process such as colloidal precipitation, sonication etc.

Ceramic core is coated by polyhydroxy compound

Loading of the drug is done by partial adsorption.

Formation of core material

The first step in the formulation of aquasomes is the development of the ceramic core. The method of ceramic core preparation depends on the choice of core materials. These ceramic cores can be built by various processes such as colloidal precipitation, sonication, inverted magnetron sputtering, and plasma condensation etc[30]. In the preparation of core; the most regular material preferred which is ceramic. Two ceramic cores that are generally used diamond and calcium phosphate.

Synthesis of Nanocrystalline tin oxide core

It can be synthesized by direct current reactive magnetron sputtering. Under a high-pressure mixture of argon and oxygen, the high purity tin is blown from a diameter of 3 inches. Ultrafine particles deposited on a copper tube in a gaseous phase and cooled to 77K with the flow of nitrogen[4].

Self-assembled Nanocrystalline brushite (calcium phosphate dihydrate)

It can be synthesized by various processes such as co-precipitation, self-precipitation, sonication and PAMAM methods.

Co-precipitation

Diammonium hydrogen phosphate solution is added drop wise to calcium nitrate solution with continuous stirring. The temperature is maintained at 75°C in a flask bearing a charge funnel, a thermometer, a reflux condenser fitted with a carbon dioxide trap[25,26]. The pH of calcium nitrate is maintained 8-10 using the concentrated aqueous ammonia solution. Under the above-mentioned condition, the mixture is magnetically stirred. The precipitates are then filtered, washed and finally dried overnight. In an electric furnace, the powder was sintered by heating to 800-900°C[35].

Sonication

Using ultrasonic bath, the solutions of disodium hydrogen phosphate and calcium chloride were mixed and sonicated. Equivalent moles of both reagents are used for 2 hrs. The temperature was maintained at 4°C. The ceramic core is separated by

centrifugation and then washed, re-suspended in distilled water and filtered. The core material retained on filter paper is collected and dried appropriately.

Poly Amido-Amine (PAMAM)

PAMAM was dissolved in the simulated body fluid of pH 7.4 and placed it for 1 week at 37°C to induce nucleation and crystal growth. By the addition of the NaOH solution, the pH of the solution was adjusted. The precipitate formed was washed multiple times with de-ionized water. Then it was filtered and dried overnight[36].

Nanocrystalline carbon ceramic, diamond particle

These ceramic may also be used for the core synthesis after ultra-cleansing and sonication. The main property of these core is crystalline[4].

Coating of the core with polyhydroxy oligomer

Commonly used coating materials are cellobiose, citrate, pyridoxal 5 phosphate, trehalose and sucrose. It is the second step in which ceramic cores are coated with carbohydrates. By the addition of carbohydrate into an aqueous dispersion of the cores under sonication, the coating is carried out. These are then subjected to lyophilization which provides irreversible adsorption of carbohydrate onto the ceramic surface. The unadsorbed carbohydrate is removed by centrifugation[35].

Immobilization of drug molecule

Loading of the drug to coated particles by partial adsorption is the final step for the preparation of aquasomes. A solution of known concentration of the drug is prepared at suitable pH buffer. Coated particles are dispersed and at low temperature, dispersion is kept at overnight for drug loading or lyophilized[38]. After sometime drug-loaded formulation obtained, then characterized using various techniques.

STABILITY PROFILE

Aquasomes basically have three layered and self-assembled structure which consist of a nanocrystalline core, carbohydrate coating and drug coating. Mainly three type of core materials are used which includes brushite, i.e., Calcium phosphate dihydrate, nanocrystalline carbon ceramics, i.e., diamonds and tin oxide. The solid core provides the structural stability to aquasomes. Calcium phosphate occurs naturally and due to its instability it gets converted in to hydroxyapatite upon prolongs storage. Owing to biodegradability, cost, stability, and safety, hydroxyapatite (HA) was selected as a core for the preparation of aquasomes. Moreover, it is widely used for the preparation of implants, and for the delivery of drugs and antigens. They are particularly suitable for protein delivery because of their high adsorption capability[38,39].

The carbohydrate coating has the property of maintaining the conformational integrity of bioactive molecules which has led to the proposal that aquasomes have potential as a carrier system for delivery of peptide, protein, hormones, antigens, genes and hydrophobic drugs to specific sites. Outer surface of aquasomes on which antigens are non-covalently linked consists of polyhydroxyl oligomers or sugar molecules. In addition to allosteric effectors such as pyridoxal-5-phosphate and sodium citrate which creates a quasi-aqueous film that prevents the denaturation or degradation of the protein. The coating also protects against dehydration and stabilizes the biochemically active molecules, e.g., Trehalose has the free bond mobility with rich hydroxyl component with a unique hydrogen bonding substrate that creates a glassy aqueous state. Other disaccharides that exhibit similar dehydroprotectant activity include cellobiose, and ironically, sucrose; other oligosaccharide-type molecules such a maltose, sorbitol, lactitol also appear to exhibit dehydroprotectant activity[39-43].

Fate of aquasome

Self-assembled aquasomes are biodegradable nanoparticles that accumulate more in liver and muscles[22]. The drug's pharmacological or biological activity can be accomplished instantly as it is detected without any surface alteration on the surface of the system and cannot find any difficulty in identifying receptor on the active site[44]. *In vivo* studies predict, biodegradation of ceramic is achieved by monocytes and multicellular cells called osteoclasts because they intervene first at the biomaterial implantation siteduring an inflammatory

reaction[45]. Two types of phagocytosis process were observed-when cells come in contact with biomaterial; either calcium phosphate crystals were taken up alone and then dissolved in the cytoplasm after the disappearance of the phagosome membrane or dissolution after the formation of the hetero phagosome[46]. Phagocytosis of calcium phosphate coincided with autophagy and the deposition of residual bodies in the cell[1].

EVALUATION PARAMETER OF AQUASOMES

Aquasomes mainly evaluated and characterized by their various morphological and structural property of their core and poly-hydroxy oligomer coating structure.

Evaluation parameter for core material

Morphological analysis and size distribution

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques are used for particle size distribution and morphological analysis[34]. To determine the particle size, samples were placed on the surface of a specimen stub coated with gold using double-sided adhesive tape in SEM while in case of TEM, particle size is determined after negative staining with phosphotungstic acid[46]. Coated core, as well as drug-loaded aquasomes are also analyzed by these techniques. Mean particle size and zeta potential of the particles can also be assessed by using photo correlation spectroscopy[27,47,48] have demonstrated the image of bovine serum albumin loaded aquasomes, using SEM that reveals a spherical shape. The prepared hydroxyapatite core revealed a size ranged between 30 and 50nm. This size was increased to be around 200nm after coating with carbohydrate, namely cellobiose that forms plain aquasomes. The size of aquasomes was further increased to be around 480nm upon loading of bovine serum albumin[48]. In another study, the size of hydroxyapatite core was found to be 90.1±2.3nm, which was increased to be ranged from 98.5±4.3 to 125.3±3.2nm according to the type of carbohydrate (oligomer) used in the preparation of aquasomes[49].

Characterization (FTIR)

Fourier transform infrared spectroscopy (FT-IR) spectroscopy used for determining structural analysis. Potassium bromide sample disk method is used[50]. Both the core and coated core can be analyzed by recording their IR spectra in the wave number range 4000-400cm-1. The characteristic peaks are observed and tally with reference peaks. Stability of the drug in the formulation can be also determined by this technique. FT-IR structural analysis revealed the characteristic peaks of ceramic core, sugar, and drug in aquasomes formulations which indicates loading of sugar and drug over the ceramic core. Moreover, FT-IR structural analysis revealed the formation of hydrogen bonds between drug and sugar[47,51].

X-ray diffraction

To study crystalline or amorphous nature of a material X-ray diffraction study is performed. The hydroxyapatite ceramic core is analyzed by exposing the core to copper (Cu), potassium (K) radiation in a wide-angle X-ray diffractometer[45]. After that the x-ray diffraction pattern of the sample is compared with the standard diffractogram, based on which the interpretations are made[50]. In a study, it was observed that calcium phosphate core, lactose individually gave identical sharp peaks for crystalline peaks but when carbohydrate coated cores were observed, peaks represented an amorphous structure[52]. It may be the reason for the coating technique (solubilization of carbohydrate insolvent and subsequent drying by lyophilization) and saturation of the surface of the core with carbohydrate[53]. Hence, diffraction study of individual components of aquasomes are done and compared to the entire aquasomes[54].

EVALUATION PARAMETER FOR COATED CORE

Carbohydrate coating

Coating of sugar over the ceramic core can be confirmed by Concanavalin A-induced aggregation method that estimates the amount of sugar coated over core. Anthrone reaction helps to estimates the residual sugar remained after coating and Phenol sulphuric acid method[47,54].

Zeta potential measurement

The adsorption of sugar over the core and the prediction of storage stability determined by the measurement of zeta potential. Some studies indicated that with the increase in the saturation process by carbohydrate on to the hydroxyapatite core, the more decrease in zeta potential value[55].

Zeta potential measures the electrostatic attraction or repulsion between particles. It is the best indicator for the stability of dispersions such as suspension and emulsion. The value of zeta potential depends on the type of carbohydrate (oligomer) used in the preparation of aquasomes. A previous study revealed that zeta potential values of aquasomes prepared from trehalose, cellobiose and pyridoxal-5phosphate were found to be -15.6±1.15, -20.4±0.9 and −23.2±1.26mV, respectively. This could be explained by the existence of a lot of electronegative atoms in the chemical structure of pyridoxal-5-phosphate, compared to trehalose and cellobiose[49]. It can also be utilized to confirm the adsorption of sugar over the core[47,48] Coating of hydroxyapatite core with cellobiose resulted in a decrease of zeta potential value from + 15.6 to −18.2mV due to the presence of numerous OH- groups of cellobiose. Zeta Potential value was further decreased to be −25.3mV upon loading of bovine serum albumin resulting from the presence of COO− groups of bovine serum albumin[58].

Glass transition temperature

Differential Scanning Calorimetry (DSC) studied used to analyses the glass transition temperature of carbohydrates and protein. DSC used to study the effect of carbohydrate on the drug-loaded to aquasomes[52] the transition from glass to rubber state can be measured using a DSC analyzer as a change in temperature upon melting of glass.

METHODS OF EVALUATION OF CARBOHYDRATE COATING[46,56]

Anthrone reaction

It is an example of the calorimetric method and used to quantify the unbound residual sugar or residual sugar remaining after coating. Anthrone reagent is added to sample and heated in a boiling water bath, cooled quickly. Under acidic conditions, carbohydrate gets hydrolyzed to hydroxymethylfurfural which reacts with enthrone reagent to yield blue green color complex. Absorbance is recorded (λmax=625nm) by using a UV-Visible spectrophotometer using glucose as standard.

Concanavalin-A induced aggregation

Used to quantify the amount of sugar-loaded on the ceramic core. Concanavalin-A solution is added to suspensions of different carbohydrate coated core (in quartz cuvettes). Absorbance is recorded in UV-Visible spectrophotometer at a wavelength of 450nm as a function of time of 5 min interval. The obtained data is subtracted from the blank experiment.

Phenol sulphuric acid method

It is also a colorimetric method used to find out total carbohydrate such as mono-, di-, oligo-, and polysaccharides present in the sample. Carbohydrate is dehydrated to furfural derivative in presence of concentrated sulphuric acid which is further reacted with phenol to produce yellow gold color.

Evaluation parameter of drug-loaded aquasomes

Drug loading efficiency

It is done to evaluate the amount of drug which is bound on the surface of aquasomes. The drug loading can be determined by incubating the aquasomes formulation without the drug in a known concentration of the drug solution for 24 hrs. At 4°C[36] after that supernatant is separated by high-speed centrifugation for 1 hrs. At low temperature in a refrigerated centrifuge[57]. Then the clear extractive supernatant is filtered and analyzed free drug content by UV spectrophotometer. The drug payload/drug loading is calculated by the following formula,

%Drug loading= (Weight of total added drug weight of the un-entrapped drug)/(Weight of aquasomes)×100

***In vitro* drug release studies**

The *in vitro* release kinetics of the loaded drug is done to study the release pattern of the drug from the aquasomes. Incubate a known quantity of drug-loaded aquasomes in a buffer of suitable pH at 37°C with continuous stirring[53]. Samples are extracted

from time to time and centrifuged for some periods at high speeds. After each withdrawal, equivalent medium volumes must be substituted. Then supernatants are analyzed to estimate the amount of released drug[57].

In-process stability studies

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) can be used to assess the stability and integrity of protein during the formulation of the aquasomes[56].

Advantages of aquasomes

Aquasomes systems act as a reservoir to release the molecules either in a continuous or a pulsatile manner, avoiding a multiple-injection schedule[58].

Aquasomes based vaccines offer many advantages as a vaccine delivery system. Cellular and humoral immune responses can be elicited to antigens adsorbed on to aquasomes[56]

Aquasomes improves the pharmaceutically active agent's therapeutic effectiveness and defends the medication from phagocytosis and degradation[56].

Enzyme activity and molecular conformation sensitivity have made aquasomes a novel carrier for enzymes such as DNAse and pigment/dyes.

Multi-layered aquasomes conjugated with bio recognition molecules such as antibodies, nucleic acid, peptides which are known as biological labels can be used for various imaging tests[58].

Other advantage of aquasomes includes the benefits like Moderate bio adhesive activity, more stable cubic vesicles, preserves integrity of protein pharmaceuticals, induce better immunological responses, specific targeting with high drug-loading molecular shielding[59].

Disadvantage of aquasomes

According to the method of preparation of aquasomes, it could be deduced that the preparation is time consuming.

The concentration of the drug solution should be carefully adjusted to not exceed a certain point at which the drug is crystallized, resulting in a false increase in the drug loading.

Care should be taken in production of carriers.

Dose dumping is carried out by carriers.

Leaching and aggregation of prolonged storage.

Expensive[25,60]

AQUASOMES IN INDIA: AN EXPEDITION TILL NOW AND THE WAY AHEAD

"Aquasomes" are carbohydrate stabilized nanoparticles of the core which was first developed by NirKossovsky in 1995[3]. Aquasomes are tri-layered self-assembled nanoparticles. They comprise a central solid immutable nanocrystalline core[61]. Though well-known in the ancient Indian traditional medical literature as Bhasmas, ceramic nanoparticles have gained wide popularity as drug delivery vehicles in the last two decades[62-64]. Apart from possessing the inherent advantages associated with nanoparticles such as large surface area to volume ratios, high payload, possibility of mass production and enhanced cellular uptake, ceramic nanoparticles offer added advantages as compared to polymeric nanoparticles. These include-prolonged release and non-swellability, leading to a largely immutable character[65]. After being successfully applied for anti-cancer drug delivery[66], photodynamic therapy[67] and bone tissue engineering[68], the second generation ceramic nanoparticles with various surface coatings have been introduced. The coating materials reported include PEG[69], metals like gold[70], peptide/ protein etc[25]. Three-layered, sugar coated ceramic nanoparticles have recently emerged as a new class of next generation therapeutic vehicles[5,71].

The history of the development of ceramic nanoparticles can be divided into three periods:

Ancient Period

Recent Period

Latest Period

Ancient period

Ayaskriti was the fine powder form of inorganic substances used in the ancient medicine as mentioned in the Charka Samhita[72]. However, variability in the fineness of the powder led to the development of new methods to ensure a reproducible fine particle size leading to formulation of Bhasmas. Nanoparticles were known in the ancient medicine as Bhasmas in Ayurvedic[62,73] and Kushtas in Unani[74] system of medicine. Bhasmas and Kushtas made up of materials like calcium salts and/ or diamond, the most commonly used material for ceramic cores for

the preparation of aquasomes, are frequently mentioned in the ancient texts as Kushta-e-kharmohra[75], Kushta-e-sadaf[76], Prawalbhasma[77], Muktashuktibhasma[78] and Vanga Bhasma[79]. The characteristic features of Bhasmas, as dictated by Ayurveda, are strikingly similar to those of the nanoparticles[63,80].

Quality control tests of Bhasmas include rekhapurnatvam, which indicates that all the particles enter into the fine lines of the fingers and are not washed off easily[81]. The particle size of the bhasma particles has been reported to be in the range of 50 nm to a few micrometers[82]. The characteristics of Apunarbhavtva (permanence) and Niruthatva (irreversiblity) of bhasmas also indicate the immutability of the characters associated with the ceramic nanoparticles[83,84].

Recent period developments

Nano-structured calcium phosphate and diamond like carbon are the most commonly used materials in ceramic nanoparticles used in varied applications such as implantable devices[85], gene delivery[86], anti-microbial activity[87], genomics, proteomics and metabolomics[88]. Drugs could be adsorbed onto the surface of the ceramic nanoparticles and selectively delivered to sites such as bones for various bone disorders[89]. Along with the uncoated ceramic nanoparticles, different kinds of surface functionalized ceramic nanoparticles have also been developed for even wider applications[90], including delivery of insulin[91], genes[92] and Boron Neutron Capture Therapy[93]. Ceramic nanoparticles coated with carious carbohydrates have also been reported as a group of glycol-fused therapeutics[94,95]. Carbohydrate-coated maghemite nanoparticles, particularly those having a coating of dextran, were found to exhibit excellent antibacterial properties[96]. Nanoparticles of hydroxyapatite, vitrified in sugar glass matrix, have been successfully investigated for sustained delivery of vaccines[97,98].

Latest period developments

Aquasomes represent the development in the field of ceramic nanoparticles, which is only a couple of decades old in modern times. They comprise a central solid nanocrystalline core, coated with polyhydroxy oligomers, onto which biochemically active molecules are adsorbed[23]. These have been studied as carriers for bioactive molecules, mainly proteins and peptides, as the carbohydrate coating protects them against dehydration and stabilizes them. The work was initiated by Kossovsky and his co-workers. In 1994, haemoglobin was adsorbed on the carbohydrate coated diamond nanoparticles and then encapsulated in a phospholipid layer[57].

Subsequently, the group successfully used these nanoparticles to deliver viral antigens and insulin[98,99]. These were proposed as potential delivery systems for protein/peptide drugs[100]. In 1996, the research group christened the particles as aquasomes based on the water like protection provided by the carbohydrate layer[101]. Since then, aquasomes have been explored for various applications. The parenteral delivery of insulin[102] and insulin-mimetic polypeptide k[103] has been reported. Aquasomal formulation, loaded with hemoglobin was prepared which was found to be stable for at least thirty days[104,105]. Their use as an adjuvant and delivery vehicle for hepatitis B vaccine for effective immunization was reported by Vyas *et al*[106]. Similarly, aquasomes loaded with BSA were designed to trigger a better immunological response[107,108]. Aquasomes of malarial merozoite surface protein (MSP) - 119 were prepared by adsorption of antigen on self-assembling hydroxyapatite carriers[109]. Aquasomes have also been used for oral delivery of enzymes like serratiopeptidase[110].

Another use of aquasomes has been demonstrated by Pandey *et al* in a study wherein allergen immunotherapy could be achieved in mice model using ovalbumin[111]. A number of synthetic drugs have also been formulated as aquasomes. These include etoposide[113], dithranol[114], piroxicam[115] and pimozide[116] etc. The aquasomal approach has been tried to overcome the common dissolution problems associated with these hydrophobic drugs. We recently examined the ability of aquasomes to interact with the most potent humoral factors in the human serum, called the complement system, which is activated via classical, alternative and lectin pathways. Activation of the complement system can influence intended therapeutic targeting and

subsequent clearance of the aquasomes by virtue of their ability to opsonize and enhance phagocytosis by phagocytic cells. The Aquasomes can potentially be seen as non-self by the recognition components of the complement system, as they do for other nanoparticles including carbon nanotubes[117,118]. Complement activation can generate anaphylatoxins such as C3a and C5a that can recruit inflammatory cells. In addition, the end-product of the complement activation, the membrane attack complex (MAC) can introduce further damage to self in the absence of available non-self-pathogens. We tested Aquasomes with calcium phosphate cores coated with mannitol via complement consumption and C1q-dependent classical pathway dependent hemolytic assays for their ability to induce classical pathway complement activation. No complement consumption was found for aquasomes up to the highest concentration tested (1mg/ml). A higher concentration of aquasomes is unlikely to activate complement and will not be clinically relevant. Although a range of aquasomes coated with other derivatives need to be examined further for complement activation via one of the pathways, these early results appear to suggest potentially non-complement activating characteristics of the aquasomes (Pondman *et al*, unpublished data). Even more interestingly, the aquasomes, when challenged against macrophages, were found to be poor inducers of pro-inflammatory cytokines such as TNF-α and IL-1β, while up-regulating the production of anti-inflammatory cytokine IL-10. Up-regulation of IL-10 may be central to anti-inflammatory response generated by aquasomes when challenged against macrophages. Clearly, a more systematic study is required to further dissect the nature of interaction between aquasomes and the innate and adaptive immune components.

Application of aquasomes in the delivery of various products reported in Table No.2. There are some active ingredients which therapeutic activity enhanced by formulating in aquasomes novel drug delivery system.

LIMITATIONS

Though aquasomes appear to be an attractive potential delivery system for a wide range of therapeutics, certain critical aspects associated with aquasomes that have not yet been studied include their amenability to various sterilization techniques, their shelf life as per ICH guidelines, feasibility of scale-up and subsequent commercialization potential, cost efficacy, reproducibility of critical parameters in terms of both *in-vitro* and *in-vivo* characterization, and most important their safety issues. All these aspects are very crucial in deciding the success of these nanoparticles and must be carried out thoroughly[75]. Another limitation is if the drug is poorly absorbed, it may cause burst release in the body that cause toxicity. To prevent opsonisation and phagocytic clearance of aquasomes in body, its surface could be coated with polyethene glycol[119].

APPLICATIONS OF AQUASOMES

Aquasomes used as vaccines for delivery of viral antigen i.e., Epstein-Barr and Immune deficiency virus to evoke correct antibody, objective of vaccine therapy must be triggered by conformationally specific target molecules.

Aquasomes as red blood cell substitutes, haemoglobin immobilized on oligomer surface because release of oxygen by haemoglobin is conformationally sensitive. By this toxicity is reduced, haemoglobin concentration of 80% achieved and reported to deliver blood in nonlinear manner like natural blood cells.

Aquasomes have been used for successful targeted intracellular gene therapy, a five layered composition comprised of ceramic core, polyoxyoligomeric film, therapeutic gene segment, additional carbohydrate film and a targeting layer of conformationally conserved viral membrane protein.

Aquasomes for pharmaceuticals delivery i.e. insulin, developed because drug activity is conformationally specific. Bio activity preserved and activity increased to 60% as compared to i.v. administration and toxicity not reported.

Aquasomes also used for delivery of enzymes like DNAase and pigments/dyes because enzymes activity fluctuates with molecular conformation and cosmetic properties of pigments are sensitive to molecular conformation[120,121].

FUTURE PERSPECTIVES

Aquasome, a self-assembled system provide a promising future in the efficient delivery of the broad range of drug molecules including viral antigens, haemoglobin and insulin. The unique carbohydrate coating of the core show better biological activity and maintain the qualities of the drug molecule and its structural integrity. Biosensors are those devices that deliver the drug or those agents which monitor the drug and help in diagnosis.

If biosensor is incorporated with the aqua some core, it may be effective to examine soft tissue in cancerous disease and also help in diagnosis. At a present time world is suffered from a pandemic COVID-19 and there is no effective line of therapy to treat this. If the concept of slow antigen release in small quantity via aqua some which produce specific antibody in the body at the sustained rate is used in case of COVID-19. It could be proven effective to enhance specific immunity against COVID19. Along with immunity loss, it also has mild symptoms such as difficulty in breathing, decreased oxygen level that could also be maintained with the oxygen transport property of aquasomes[1].

Table No.1: Various investigations on aquasomes as vesicular drug delivery system

S. No.	Formulators	year	Drug	Experiment	Therapeutic Use
1	Nanjwade et al.	2013	Etoposide	Etoposide aquasomes were obtained through the formation of an inorganic core of calcium phosphate covered with a lactose film and further adsorption of the etoposide. The diameter of drug-loaded aquasomes was found to be in the range of 150–250 nm. Entrapment efficiency was found to be 88.41%. The average targeting efficiency of drug-loaded nanoparticles was found to be 42.54% of the injected dose in liver, 12.22% in lungs, 4.14% in kidney, and 25.12% in spleen. The results discovered that the nanoparticles-bearing drug showed better drug targeting to liver followed by spleen, lungs, and kidney	Anti-Cancer
2	Vengal et al.	2013	Piroxicam	Ceramic nanoparticles of poorly aqueous soluble piroxicam were prepared to explore the relationship between particle size and dissolution profile. The %yield of ceramic nanoparticles was 66.7%. It was observed that the piroxicam ceramic nanoparticle formulations elicited release of piroxicam in 1 h and 15 min	Painkiller(NSAID)
3	Tiwari et al.	2012	Dithranol	Aquasomes were developed employing colloidal precipitation process and were spread into a cream for psoriasis treatment. The drug-loading efficiency was found to be 84.8% w/w. 55.93% drug release was observed in 7 h. In vitro drug release studies from both the creams revealed that aquasome-loaded cream controlled the drug release as compared to plain cream	Psoriasis Treatment
4	Kommineni et al.	2012	Insulin	Insulin-bearing aquasomes were prepared by the standard method employed for the preparation of aquasomes. The in vivo data of the framed aquasome was associated with standard porcine insulin solution, and promising results were obtained in comparison to insulin solution	Anti-diabetic
5	Cherian et al.	2000	Piroxicam	Ceramic nanoparticles were developed by coprecipitation by refluxing and sonication. Core preparation was finalized sonication method, grounded on the high %yield (42.4 ± 0.4%) and smaller period (1 day) equated to the reflux method (27.4 ± 2.05%, 6 days). Morphological evaluation revealed spherical nanoparticles (size 56.56 ± 5.93 nm for lactose-coated core and 184.75 ± 13.78 nm for piroxicam-loaded aquasomes) confirming the nanometric dimensions	Osteoarthritis Treatment

Table No.2: Delivery of various products via aquasomes approach

S.No	Active Ingredient	Therapeutic Application	Aquasome-drug complex result outcome	Formulation	Reference
1.	Dithranol	Treatment of psoriasis	Controlled drug release	Topical (Cream)	133
2.	Insulin	Glucose regulation	Controlled drug release	Parenteral	21
3.	Serratiopeptidase	To improve proteolytic activity	Sustained release profile	Oral	132
4.	Etoposide	Anti-cancer targeting	Drug targeting	Oral	134
5.	IFNα	Anti-tumor	Prolonged release profile		47
6.	Haemoglobin	Blood component, the oxygen carrier	Toxicity is reduced and increase stability	As RBCs substitute carrier	28
7	Hepatitis B vaccine	Antigen for prevention of jaundice	Effective immunization	Parenteral	33
8.	Serum Albumin	Maintain osmotic pressure, needed for proper distribution of body fluids between intravascular compartments and body tissues	Better immunization response		135
9.	MSP119 (Merozoitesr face protein)	Anti-malarial	Better absorption efficiency		20
10.	Indomethacin	Anti-inflammatory	Sustained release of drug		36
11.	Mussel Adhesive Protein	Antigen delivery	Antigen stabilization		29
12	Non-nuclearsubstancefromHIV-1	Immunization	Elicit both humoral and cellular immunity		107
13	Insulin	Insulin delivery	Protection of peptides, Enhanced Insulin delivery		21
14	Hemoglobin	Oxygencarrier	High potential as artificial blood substitute		46
15	Hemoglobin	Hemoglobin carrier	Stable formulation having up surged oxygen carrying capacity		114

16	Hepatitis Bserum Antigen	Vaccine delivery	Eliciting combined immune response		51
17	Indomethacin	Dissolution enhancement	Developed indomethac in aquasomes and structurally analysed		36
18	Bovine serum albumin	Antigen delivery	Structurally preserved BSA and improved presentation to Antigen Presenting Cells		116
19	Ovalbumin (OVA)	Allergen immunotherapy	Induces a strong T cell specific proliferative response (Th1) without abrogation of Th2 responses		45
20	Piroxicam	Oral delivery of hydrophobic drugs	Enhanced oral delivery of piroxicam and reduces the systemic side effects		48
21	Piroxicam	Lipophilic drug use	Controlled release of piroxicam		47
22	Pimozide	Dissolution improvement of hydrophobic drug	Improved dissolution profile of aquasomes		47
23	Etoposide	Dissolution up surging of poor soluble drug	Highest % of the dose was found in spleen and liver		137
24	Interferon	Protein delivery	Protein delivery		60
25	Lornoxicam	Lipophilic drug delivery	Enhanced the dissolution with better release profile		137

Table No.3: Some recent patents on aquasomes drug products

S.No	Patent Title with its application	Author's Names	Publication no	Publication year
1	Acoustic field coupling with micro-devices	Freitas JR Roberta, Hogg tad	US10024950B1	2018
2	Topical compositions for stimulating hair Growth	Brichtlalars	W02019236596A1	2019
3	Gel formulation for treating diabetic foot ulcer	Uma Shankarmarakanam, Srinivasan	US2020188314A1	2020

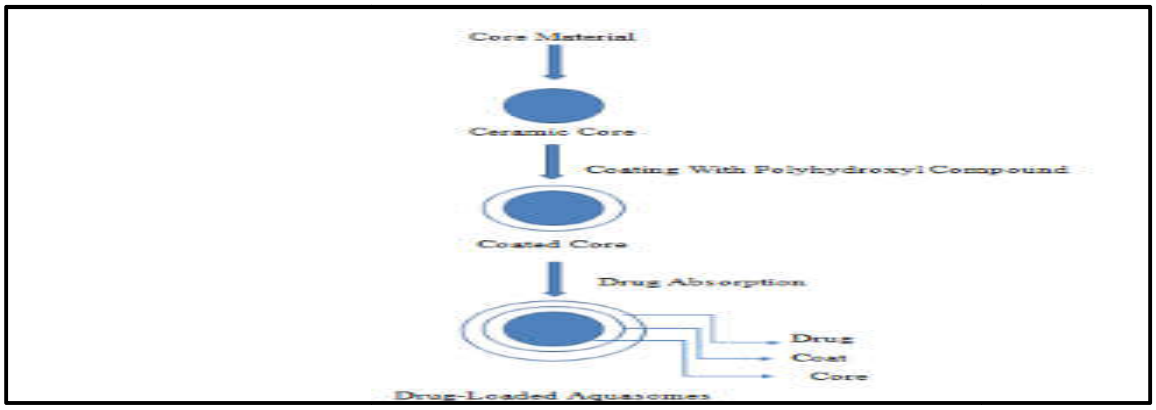

Figure No.1: Schematic diagram of aquasomes

CONCLUSION

Aquasomes showed higher ability to produce enhanced immune response. So, aquasomes based strategy relishes for the delivery of a wide range of bioactive molecules and in the effective possible treatment of various disorders. Aquasomes as the potential carriers capable of preserving the structural integrity, may bring a new transformation in the field of protein and peptide delivery in protein pharmaceuticals. Furthermore, these can be used as immune adjuvants for protein antigens as they evoke a better immunological response. Also, they are using as a better option to tackle the various limitations of the conventional dosage forms because they are having a great future potential applications as a carrier system. However, a diligent study of pharmacokinetics, toxicology, animal studies to validate the aquasomes safety, efficacy and the other regulatory criterion in contemplation of their clinical advantageousness and commercial prospects.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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