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Review Article

Virosomes : New Frontier for Targeting Drugs and Biological Molecules

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ABSTRACT

Over the last decade, there has been a great revolution in drug delivery technologies. Delivery of drugs and biological molecules using virosomes is a novel technique available these days. Virosomes are the unilamellar phospholipid bilayer vesicles containing virus derived proteins. They show the targeted action by fusing with the target cell. They may be used as vaccines or vehicle for cellular delivery of macromolecules. Along with the delivery of these substances, it also helps in potentiating immune response. The success of virosomal drug delivery depends upon the method of preparation, use of proper detergents and short chain phospholipids, and function of hemagglutinins. Several parameters are studied regarding their morphology, safety, efficacy and stability. It is also observed that virosomes are showing greater target specificity in combating ebola infection. Though it is a novel, effective drug delivery mechanism, advanced research is needed to use virosomes as a powerful tool.

Key-words: Virosomes, Hemagglutinin, Neuraminidase, Detergents, Drug delivery systems.

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1. INTRODUCTION:

The major disadvantage of most of the promising drugs is failure to achieve targeted action. The conditions like cancer and many neurodegenerative disorders require targeted and controlled release actions. Promising drugs are often discontinued during development because of their inability to achieve targeted action. [1] To avoid such systemic toxicity, novel, sophisticated targeted drug delivery mechanisms are needed. Virosomes are the semi synthetic complexes derived from nucleic acid free viral particles. It is a drug or vaccine delivery mechanism consisting of unilamellar phospholipid bilayer vesicle incorporating virus derived proteins to allow the virosomes to fuse with target cells. They are not able to replicate but are 'Fusion Active Vesicles' also known as 'Virus like Particles'. [2] Virosomes retain their fusogenic activity and shows the targeted action of incorporated pharmaceuticals or biological materials like antigens, drugs, genes etc. Therefore they can be used as drug delivery materials, vaccines or gene carriers. Pharmaceutically active substance incorporated within virosome, is protected from proteolytic degradation and low pH within endosomes, which allows them to remain intact till it reach the cytoplasm. [3] Virosomes were first prepared by Almeida *et al* where they inserted purified influenza spike proteins into preformed liposomes.[4] Influenza virus is the most common virus of choice where obtained virosomes are devoid of inner nucleic acid core and genetic information. Thereafter a range of viral envelopes have been reconstituted. Following are some examples of viruses used for the virosome preparation:

Sr. No	Virus used for virosome formation	Reference
1.	Sendai virus	5,6,7
2.	Semliki Forest virus (SFV)	8, 9
3.	vesicular stomatitis virus (VSV)	10,11
4.	Sindbis virus	12

Table No 1: Virus used in virosome production

2. NEED FOR DEVELOPMENT OF VIROSOMES: [1, 2]

2.1 To achieve targeted action-
Virosomal system should be developed to avoid systemic side effects and to achieve site specific action.

2.2 To assist delivery of macromolecules-
Delivery of macromolecules is the most important factor in therapy. As virosomes have inner vacant space, they can be used for macromolecule targeting.

2.3 To protect pharmaceutically active substance from degradation-
Degradation of active ingredient generally occurs in the stomach after oral ingestion. Also proteolytic and degradation due to low pH of endosomes is of concern. Use of virosomes can avoid this problem.

2.4 To decrease side effects-
With the traditional systems, targeted action cannot be achieved which ultimately results in increased toxicity. To overcome this factor, virosome development is necessary.

3. ADVANTAGES OF VIROSOMAL DRUG DELIVERY: [2, 9 13, 14]

3.1 FDA approved technology for use in humans and has a high safety profile.
3.2 No disease transmission risk
3.3 Non- toxic, biodegradable, biocompatible
3.4 No autoimmunogenesity or anaphylaxis
3.5 Almost all important moieties can be incorporated (Anticancer drugs, proteins, peptides, genetic materials, antibiotics, fungicides)
3.6 Assists drug delivery to the targeted cell
3.7 Protects drug against degradation
3.8 Easy amplification of immune response
3.9 Allows conformational stabilization of protein and peptide antigen even of barely soluble antigens.
3.10 Strong immunostimultion via T helper cells
3.11 Suitable for population like elderly, infants and immunosuppressed

4. DISADVANTAGES: [15, 16]

Different problems have been encountered during virosomes development

4.1 Shorter shelf life

4.2 Manufacturing problems

4.3 Poor quality of raw materials

4.4 Non availability of data related to the chronic use of virosomal carrier system.

Remedial measures have been taken to overcome these issues [17] which includes,-

Use of products with high quality and improved purification protocols, use of improved validation analytical techniques, determination of batch to batch variability with quality control assay using sophisticated instruments, use of remote loading techniques to overcome payload problems, improvement in shelf life using appropriate cryoprotectants and lyoprotectants. Scale up problem can be improved by selection of proper method of preparation and sterilization (autoclaving or membrane filtration) coupled with aseptic and validated pyrogen removal Lal test.

5. STRUCTURE OF VIROSOMES:

Virosomes are spherical unilamellar vesicles. The main difference between virus and virosome is that, virosomes are devoid of genetic materials and non-membrane proteins as shown in figure No 1. They have mean diameter of approximately 150 nm. [18]Influenza virus is often is used for the preparation of virosomes.

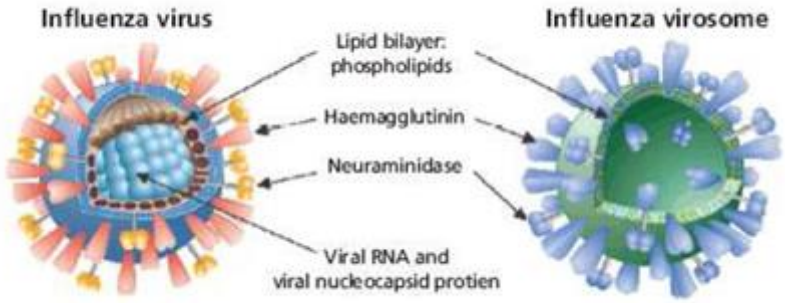

Figure No 1: Difference between Influenza Virus and Virosome

They contain functional viral envelope glycoproteins intercalated within the phospholipid bilayer membrane. [19] These glycoproteins are-

A. Influenza virus Hemagglutinin (HA) and
B. Neuraminidase (NA)

Envelope of a virus is dissolved using detergent or short chain phospholipids. Upon removal of these compounds, viral membrane is reconstituted, producing a virosome. Final virosome contains viral membrane proteins and lipids. The HA and NA proteins of virosomes facilitate efficient vesicle uptake by subsequent activation of cells of the immune system. [20] NA is involved in viral budding. [21] Depending upon whether positively or negatively charged phospholipids loaded, ligands such as cytokines, peptides, and monoclonal antibodies (MAbs) can be incorporated into virosome or displayed on the virosomal surface. Linking of tumor specific monoclonal antibody fragments (Fab) is also possible either directly or with the help of carriers. [1]

6. FUNCTION OF INFLUENZA VIRUS HEMAGGLUTININ (HA):

HA is the major spike protein which assists binding and membrane fusion. It is a class I fusion protein and associates into trimers. The precursor of Hemagglutinin i.e. HA_0 gets cleaved into HA_1 and HA_2 which are linked by two disulfide bonds. When influenza encounters a target cell, virus HA1 binds to sialic acid residue on the cell surface. It enters in the endosomal pathway where acidic pH within the endosome

induces a conformational change in HA. N-terminal fusion peptide of HA_2, which was buried in the stem of HA trimer, gets exposed and inserted into into endosomal membrane. Consequently protein refolds into hairpin structure which brings viral membrane and target membrane in close proximity thereby inducing fusion. [22, 23, 24, 25, 26]

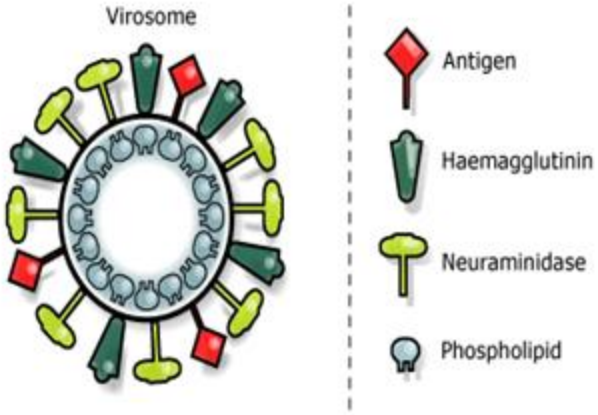

Figure No 2: Different Components of Virosomes

7. DETERGENTS AND SHORT CHAIN PHOSPHOLIPIDS USED IN VIROSOME PREPARATION:

Successful delivery of therapeutically active ingredient with virosomes depends upon proper reconstitution of membrane proteins (helps in improvement of immunogenic properties, receptor binding and membrane fusion activity). Detergents like octaethyleneglycol mono (n- dodecyl) ether ($C_{12}E_8$). After reconstitution of viral membrane, removal of $C_{12}E_8$ is accomplished through adsorption onto a hydrophobic resin (like Bio- Beads SM-2). Other detergents used frequently in the viral membrane reconstitution are Triton X- 100, octylglucoside (OG).

Short chain lecithins – from the class of ampiphilic molecules shows detergent like properties and it was first observed by Gabriel and colleagues in 1984. [27, 28] Short chain lecithin 1, 2 diheptanoyl -sn- glycerol 3-phosphocholine (DHPC) is used for various studies. But due to low Critical Micelle Concentration (CMC), 2 mM, its removal is difficult. It was observed that, 1, 2 dicaproyl- sn- glycerol- 3 phosphocholine (DCPC) would be appropriate for membrane reconstitution. [21]

8. MECHANISM OF ACTION:

Hemagglutinins are arranged repetitively on the virosomal surface which allows easy interaction of immunoglobulin receptors present on B lymphocytes. [29, 30] Virosomes are taken up by dendritic cells (antigen presenting cells). Both the antigens on the virosomal surface and that derived from degraded virosomes enters MHC class II pathway, activating T helper cells. Antigen, through fusion of virosome, access the cytosolic MHC class I pathway, activating cytotoxic T lymphocutes (CTL) as shown in figure no. 3, Ligands can be attached on the virosomal surfaces to achieve targeted action like drugs having lower therapeutic window (cytotoxic drugs).

Virosomes mainly act by two ways- [31, 32 and 33]

8.1 By stimulating immune response-
- 8.1.1 Carrier function
- 8.1.2 Adjuvant function

8.2 By delivering drug to the site of action-
- 8.2.1 Entrapment of drug
- 8.2.2 Adsorption on virosomal surface

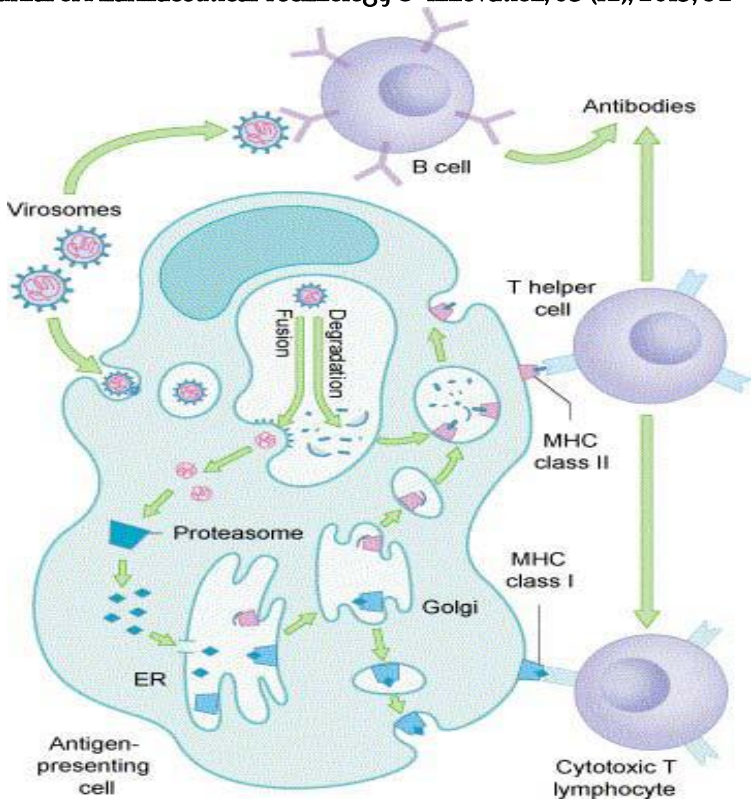

Figure No 3: Interaction of Virosomes with Cells of Immune System

9.1 Stimulation of immune response-

Virosomes acts as carrier as well as adjuvant to induce immune response. They have succeeded in stimulating specific immunity without any non-specific inflammation.

9.1.1 Carrier function-

Carrier function shows positive effect of embedded antigen. Integration of antigen into virosome

- Stabilizes antigen.
- Preserves native status of B cell epitope.

The antigen on the surface of virosome mimics the original pathogen or target cell. It generates the antibodies against specified condition. When antigens are incorporated onto virosome surface, they enhance the recognition of antibody producing B cells. Such complexes are attractive target for immune cells, which is an important step in immunity induction.

9.1.2 Adjuvant function-

Adjuvant function is based on the presence of hemagglutinin. In case of influenza derived virosomes, pre-existing antibodies against influenza bind to virosome and tag them. It initiates the uptake of this complex by Antigen Presenting Cells (APC). Now APC displays processed fragments of influenza protein. After processing by APCs, pre- existing influenza specific helper T cells are activated which are consequently proliferated and enhanced the induction of immune cells.

9.2 By delivering drug to the site of action-

9.2.1 By entrapment-

Drug can be entrapped inside the virosome. Particularly they are useful in delivery of genes, nucleic acid, enzymes and peptides etc. as shown in figure no 4. Incorporation of these components is done at the time of virosome preparation.

e.g.: The gelonin subunit 'A' of diphtheria toxin and ovalbumin has been successfully delivered by virosomal system to target cells. [34]

Following steps are involved in the delivery of active moiety-

i. Binding of virosome with membrane of target cell

ii. Disruption of cell membrane
iii. Fusion of virosome bilayer with cell membrane
iv. Intracellular delivery of virosome content

9.2.2 By adsorption-

Pharmaceutically active ingredient is either attached directly or adsorbed onto the surface of virosomes using carrier.

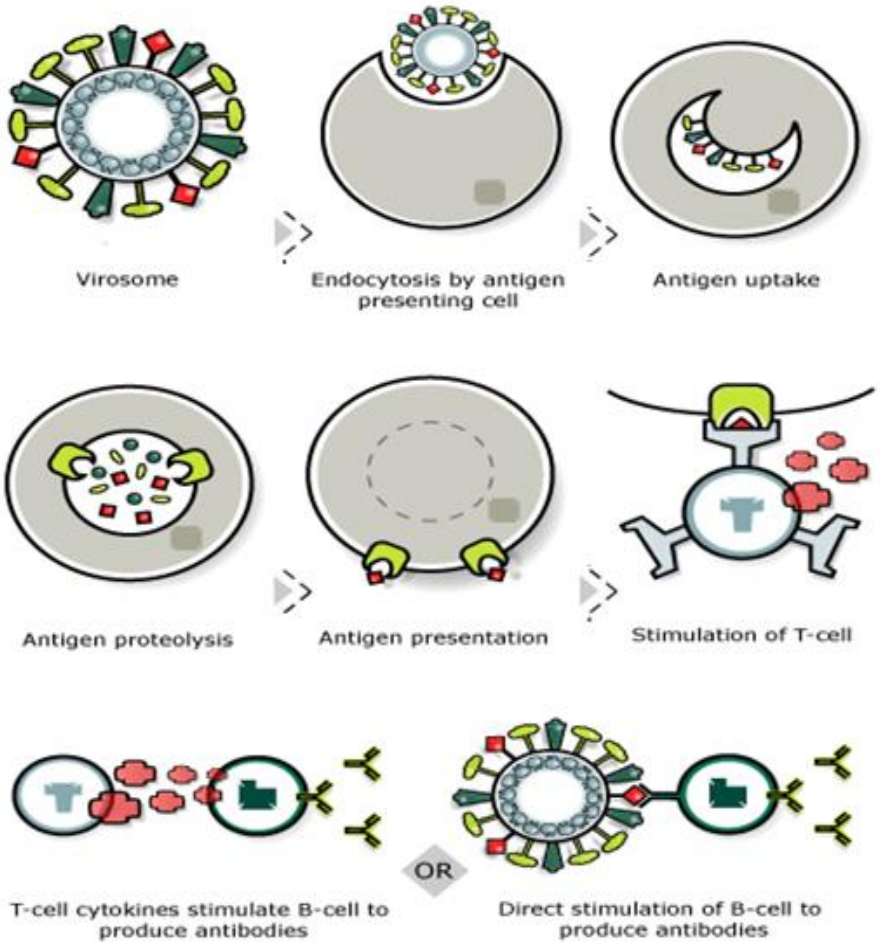

Figure No 3: Carrier and Adjuvant Function of Virosome

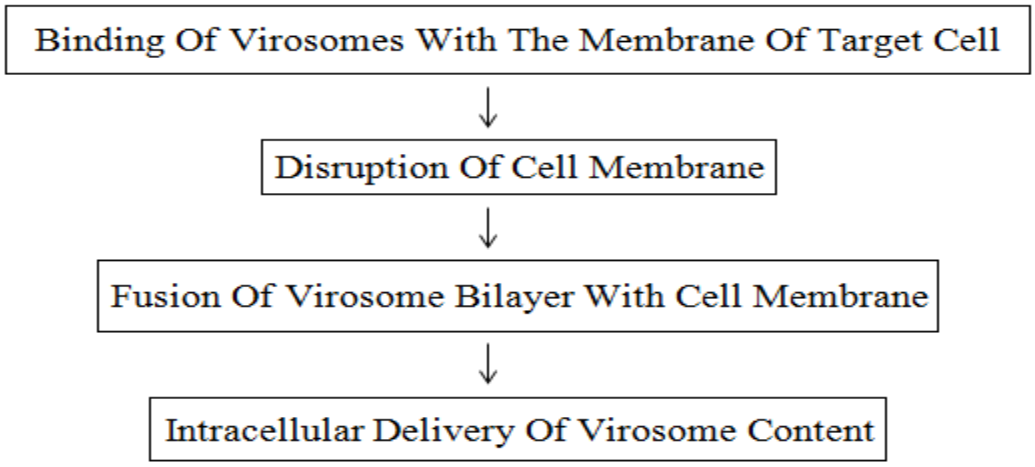

Figure No 4: Mechanism of Drug Delivery by Virosomes.

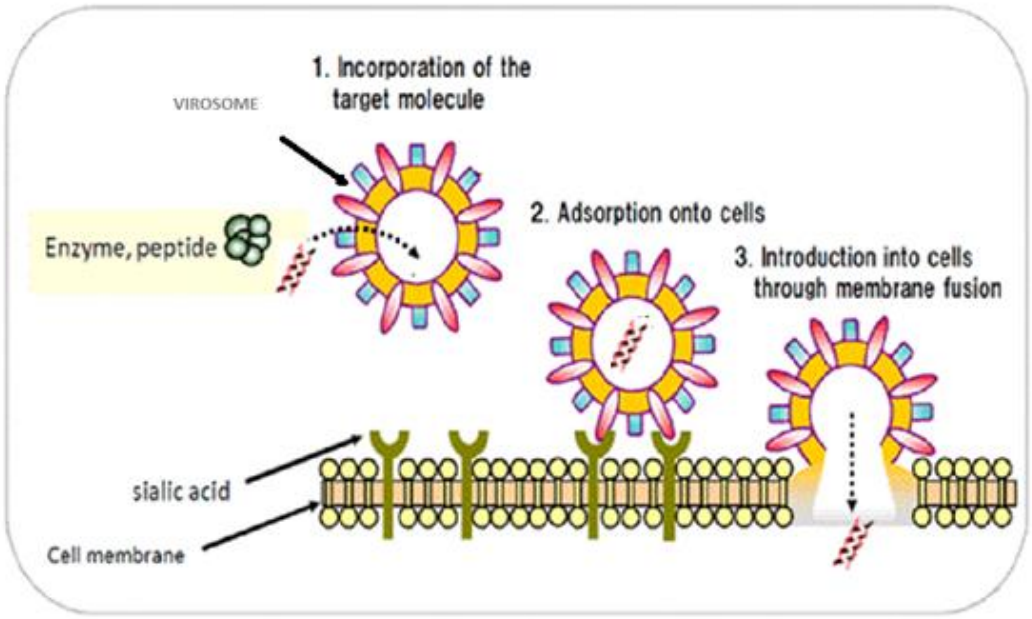

Figure No 5: Delivery of active moiety by Entrapment Process.

9. PREPARATION OF VIROSOMES:

9.1 General steps involved in virosome preparation:

9.1.1 Selection of virus-

In most of the cases, Influenza virus envelope is used to produce virosomes. Virosomes can also be made from Sendai virus, Epstein- burr virus, Herps simplex virus, Newcastle disease virus etc.

9.1.2 Selection of antigen-

Antigen may be a parasite, carcinogenic cell or bacterium. [2] Cell components can also be used like DNA, RNA or plasmid. Antigens are either directly attached or coupled with the anchor for loading onto virosome.

9.1.3 Reconstitution of virosome-

Selection of detergents and short chain phospholipids is based on the critical micelle concentration of the same. Detergents like octaglucoside, triton X- 100, ninidert p- 40 are used for solubilization purpose. It causes sedimentation of \internal viral protein and genetic material. Detergents are then removed from supernatant by different methods like dialysis, using hydrophobic resins. Critical micelle concentration of these detergents plays a vital role in virosome formation. Ultracentrifugation process removes viral matrix proteins and nucleocapsids whereas viral phospholipids are recovered. [21] Antigen to be attached is mixed with polymer or surfactant solution for binding with virosome.

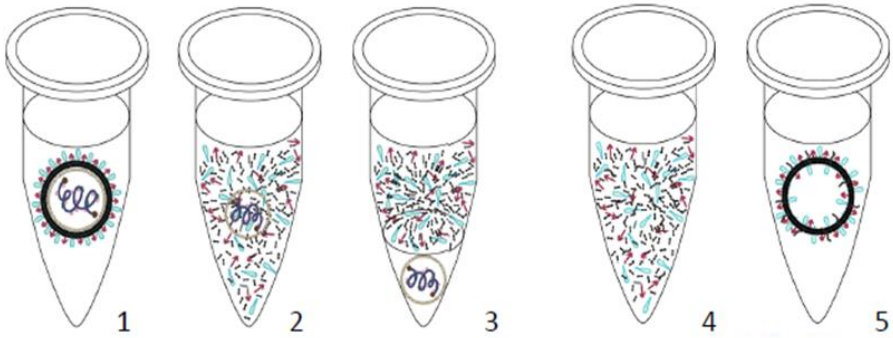

Figure No 6: Preparation of Virosomes: 1. Purified virus 2. Solubilization 3. Removal of nucleic acid 4. Addition of ligands 5. Removal of detergent & membrane reformation

9.2 Preparation of virosomes for drug delivery:

Incorporation of viral functions into liposomes is an effective strategy in the virosomal drug delivery. A new approach of use of cationic lipids was reported recently. Influenza virus A was solubilized with detergent and mixed with a cationic lipid, dioleoyldimethylamonium chloride (DODAC), 30%, to form a cationic virosome. Plasmid DNA was complexed with cationic virosome and transferred to the cultured cells by low pH dependent membrane fusion. [35] This feature serves attractive for cytoplasmic drug delivery after efficient encapsulation of respective compound. This concept has been described by Waelti et al [36]. They have investigated inhibition of tumor progression in a mouse model after treatment with a virosomal formulation. Formulation included phosphatidylethanolamine-PEG-an-chored antibodies for targeting and HA for cytoplasmic delivery of the encapsulated doxorubicin. Virosomes deliver the drug into cytosol of the cell by receptor mediated endocytosis and endosomal fusion. This system allows cell specific therapy. [36]

9.3 Preparation of virosomes for Antigen Delivery (Vaccine Delivery)-

Use of virosomes for delivery of antigens has been investigated for several diseases like malaria, melanoma, and hepatitis C virus and Alzheimer disease. [37] Studies have shown that virosomes effectively induce antibody response against conformational epitopes using cyclic- template bound peptidomimetics. Unrelated antigens can also be incorporated onto virosomal membrane. For example, Glqck et al have incorporated hepatitis A virion (HAV) in influenza derived virosomes which showed the strong stimulation of HAV specific antibody response 23. Epaxal vaccine which is currently on the market is virosomal HAV vaccine.

Fusion active influenza virosomes have ability to efficiently deliver encapsulated fragment A of diphtheria toxin (DTA) to the cytosol of cells.[38] Virosomes have capacity to deliver antigens to the MHC class I pathway in vivo and it can also activate antigen specific CTLs. This was first assessed by immunizing the mice with influenza virosome containing a synthetic epitope from influenza NP40.

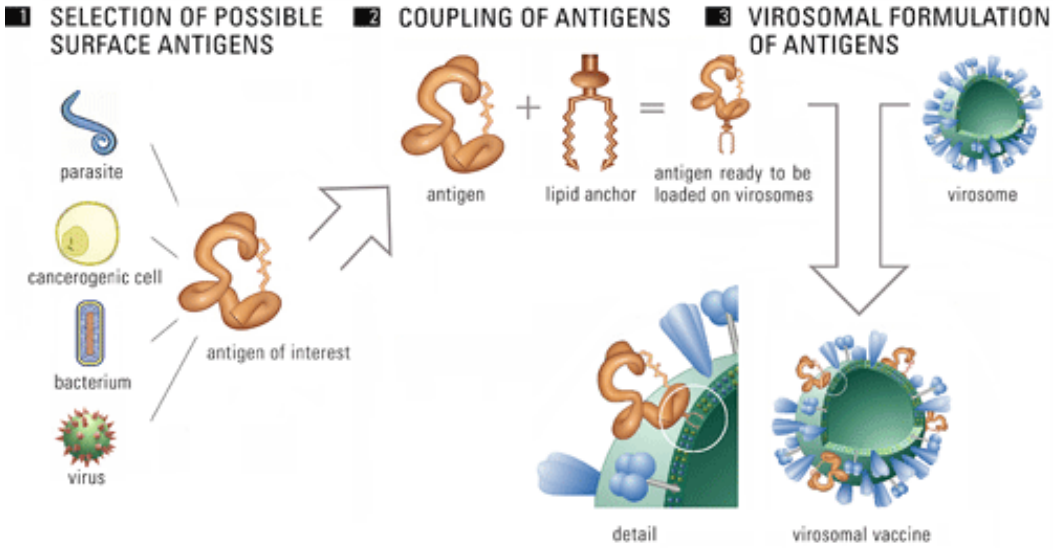

Figure No. 7: Virosome Based Vaccine Design

10. ADMINISTRATION OF VIROSOMES:

Different routes can be used for the administration of virosomes. They can be given intravenously, intramuscularly, intra-arterial. It can also be given by subcutaneous way. Inhalation delivery is possible using virosomes. They are given orally, topically, transdermally. Virosomes can be developed into an implantable device for long term release. [39]

11. PHARMACOKINETICS OF VIROSOMES:

Pharmacokinetic studies will give the information regarding differences in the pharmacological effect of entrapped drug and free drug and it help in dose designing. Study of virosomal pharmacokinetics includes time of absorption, distribution and degradation of virosomal carriers in vivo. It also needs thorough

information regarding sites of administration (mostly intravenous). Alterations are observed in both the tissue distribution and the rate of clearance of drug. Drug is carried within the virosomal aqueous environment during circulation under optimized conditions and leaks out at the sufficient rate to become bioavailable. In this case, bioavailability can be expresses as the amount of drug that is able to escape the confines of the carrier and thus become bioavailable for redistribution to the neighboring tissue. Effect of pharmacokinetic parameters on virosomes showed following outcomes - [40, 41]

11.1 Higher therapeutic index

11.2 Decrease in amount of toxicity

11.3 Decrease in occurrence of nonspecific reactions

11.4 Protection of drug from metabolism and inactivation in plasma

11.5 Reduction in non- specific localization (decreased V_d)

11.6 Increased concentration at the targeted site

12. CHARECTERIZATION OF VIROSOMES:

12.1 Protein detection:

Relatively uniform protein- to- lipid ratio is observed in prepared virosomes. To confirm the presence of HA protein, Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS- PAGE) method is used. [39]

12.2 Structure and size-

Negative stain electron microscopy with preferably neutral staining agent solution is used to determine the structure and size of virosomes. Neutral pH is used to avoid acid induced conformational changes in HA protein. [42] Dynamic light scattering, transmission electron microscopy, photo correlation spectroscopy, laser light scattering can also be used to determine vesicle size and size distribution

12.3 Fusion activity:

Membrane fusion activity of virosomes is pH dependent. *In vitro* fusion can be assessed with biological or artificial target membranes- excimer assay. In this assay, pyrenes labeled lipids are used where decrease in excimer fluorensence is due to decrease in surface density of pyrene- phosphatidylcholine- label on fusion with unlabeled membrane.

12.4 Surface charge:

Free flow electrophoresis techinique is used to determine the surface charge

12.5 Lamellarity:

It can be studied using X- ray scattering, freeze fracture electron microscopy etc.

12.6 Percent free drug:

Mini column centrifugation, gel exclusion, protamine aggregation, and radiolabel ling.

12.7 Pyrogenicity:

Rabbit fever response test or limulus ambeocyte lysate **(**LAL) test.

12.8 Surface chemical analysis:

Static secondary ion mass spectrometry.

13. APPLICATIONS OF VIROSOMES:

13.1 Cancer treatment:

In oncology field, virosomes are used to carry peptides like Tumor Associated Antigens (TAA).
e.g.: virosomes with peptides derived from parathyroid hormone related protein (PTH- rP) [43]

13.2 Gene delivery: [44. 45]

It is observed that, HA mediates the low pH dependent fusion activity followed by cellular uptake of virus particles by receptor mediated endocytosis. This activity of HA shows efficient gene delivery. [46]

13.3 RNA/ DNA Delivery-

Downregulation of newly induced proteins is assisted by encapsulating small interfering RNA (siRNA) in virosomes. It overcomes the drawbacks of other methods. [47, 48]

13.4 Malaria therapy-

Virosomes formulated with antimalarial peptides shows good tolerability and high specific immune response in humans. Scientists have identified two peptide structures which serve as antigens for malarial vaccines: The NPNA region and merozoit apical membrane antigen- 1 (AMA- 1). [49, 50]

14. EBOLA VIROSOMES:

Ebola virus disease (EDV) or simple ebola is a disease of humans and other primates caused by ebola virus. In March 2015, the World Health Organization (WHO) reported a major ebola outbreak in Guinea, a western African nation. It is a deadly infection which shows sever hemorrhage and ultimately death if not treated properly. [51, 52] Ebola virosomes were produced in insect cells using a recombinant baculovirus expression system and their efficacy against EVD was investigated. Ebola glycoprotein 1, 2 (GP1, 2) is the active antigen. Immunization of mice with MLD deleted GP (GPΔMLD) virosome conferred complete protection of vaccinated mice against ebola infection. [53]

15. VIROSOMAL PREPARATIONS: [4, 39, 42, 49, 50, 54]

Sr. No	Virosomal Preparations	Applications
A.	**Antigen Based Products**	
1.	Hepatitis A virus envelope proteins (Epaxal)	Hepatitis A
2.	Influenza virus	Influenza
B.	**Preparations under clinical trials**	
1.	Diphtheria/ Tetanus toxoid virus envelope proteins	Diphtheria, Tetanus
2.	Protein of plasmodium falciparum AMA- 1	Malaria
3.	PEV	Breast cancer
C.	**Preparations under preclinical trials**	
1.	Doxorubicin	Cancer
2.	DNA encoded mumps antigen	Mumps
3.	Hepatitis C peptides	Hepatitis C

Table No 2: Preparations of Virosomes

Sr. No	US Patent No.	Title
1	7576066	Nucleic acid compositions for stimulating immune responses
2	7615227	Use of CpG oligodeoxynucleotides to induce angiogenesis
3	7615377	Fluorescein-based metal sensors
4	7615539	Nucleic acid-lipophilic conjugates
5	7618641	Functionally reconstituted viral membranes containing adjuvant

Table No 3: Recent US Patents Of Virosomal Drug Delivery

16. CONCLUSION:

Virosomal drug delivery is an innovative approach for targeting drugs and various biologically active molecules, specially nucleic acids or genes. However extensive study is required regarding pharmacokinetic profile, bioavailability and clinical effects. Also stability parameters should be studied to assure their long term reliability, safety and efficacy.

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