Applications of viral nanoparticles

This section deals with the surfactant-based systems and their requirements for effective gene delivery. A discussion on virosomes is also included.

1 Surfactant-assisted oligonucleotide complexation

Surfactants are amphipathic molecules that possess hydrophobic and hydrophilic segments. These molecules can self-assemble in to micelle structures above their critical micelle concentration (CMC) when introduced into an aqueous phase. There are three categories of surfactants – cationic, anionic and non-ionic. The cationic surfactants can be used to complex anionic oligonucleotides in a cooperative self-assembly process.The formation of the self-assembled structures is much more accelerated in the presence of both components when compared with the individual species. Nearly monodispersenano-dimensional complexes are formed with good complexation efficiency rapidly. One of the prominent members of the cationic surfactant family is Cetyltrimethyl ammonium bromide (CTAB).Figure 1 shows the structure of CTAB.

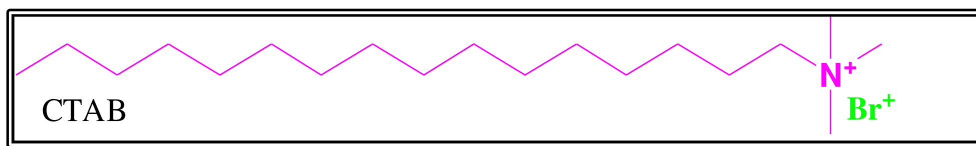

Fig. 1: Structure of CTAB

CTAB readily forms complexes with oligonucleotides. However, when it is diluted or introduced in a system containing phospholipid bilayer such as liposomal vesicles or cells, then the surfactant dissociates fromthe oligonucleotide complex and incorporates with the lipid bilayer. This results in destabilization of the oligonucleotide-surfactant complex and the oligonucleotide is released even before it enters the cell! Hence, it is essential that the surfactant system should be designed in such a manner to enhance its stability even in presence of surfactant traps such as the lipid bilayers. The development of '**Gemini**' surfactants provides an ideal solution to the stability of the oligonucleotide-surfactant complexes.

1.1 Gemini surfactants

A Gemini surfactant contains a polar head group and a hydrophobic acyl chain and hence is an amphiphile. What makes it different from conventional surfactants is that they possess a sulphydryl or thiol (-SH) group. How does this make them unique? On introduction in to an aqueous medium, these surfactants tend to self-assemble to form micelles. This brings the thiol groups in close proximity to each other and they spontaneously oxidise to form a disulphide bond between the two amphiphiles. The covalent bond confers stability to the structure thus retarding the disruption of the

oligonucleotide-surfactant complex. The structure of the oxidized surfactantcontains two identical hydrophobic domains and identical polar head groups interconnected through a disulphide bridge thus making them 'twins' or more precisely conjoined twins! As in the zodiac, the sun sign 'Gemini' is represented by twins, these thiol containing surfactants are referred to as 'Gemini' surfactants. Figure 2 shows the formation of a typical Gemini surfactant.

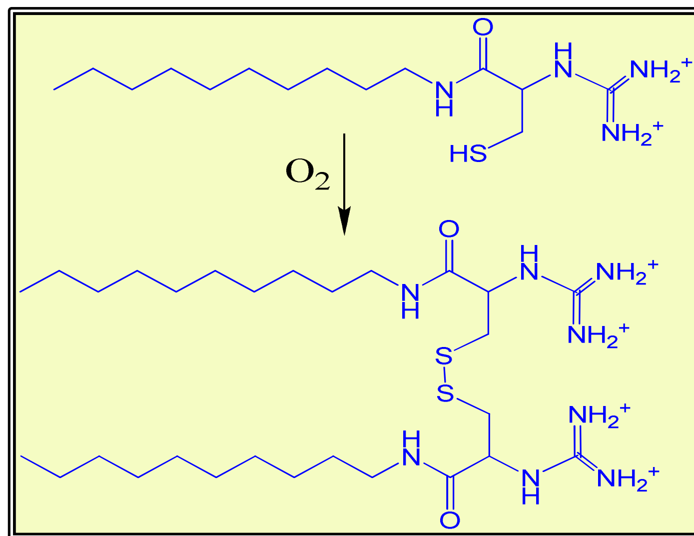

Fig.2: Oxidation of a thiol containing surfactant to form a Gemini surfactant

If the polar head group contains a cationic moiety, then the complexation of oligonucleotides will be possible. What is interesting is that each Gemini structure will contain two identical cationic head groups and hence double the positive charges when compared to a single cationic surfactant. This provides an added advantage of faster and more effective oligonucleotide complexation. Indeed, the critical aggregation concentrations for the Gemini surfactant-oligonucleotide complexes are much lower than those observed with conventional cationic surfactants.

One of the important criteria that influences the complexation efficiency and stability of Gemini surfactant based gene delivery systems is the number of positive charges present in the head group. The initial Gemini surfactants were developed with a guanidino groupwhich when in the Gemini state contained two positive charges. Later, homologues were developed with ornithine as the charged moiety. The resultant Gemini structure had four positive charges. When ornithine was substituted with the tetra-positive spermine moiety, the resultant Gemini structure contained eight positive centres. Figure 3 shows the structure of some Gemini surfactants.

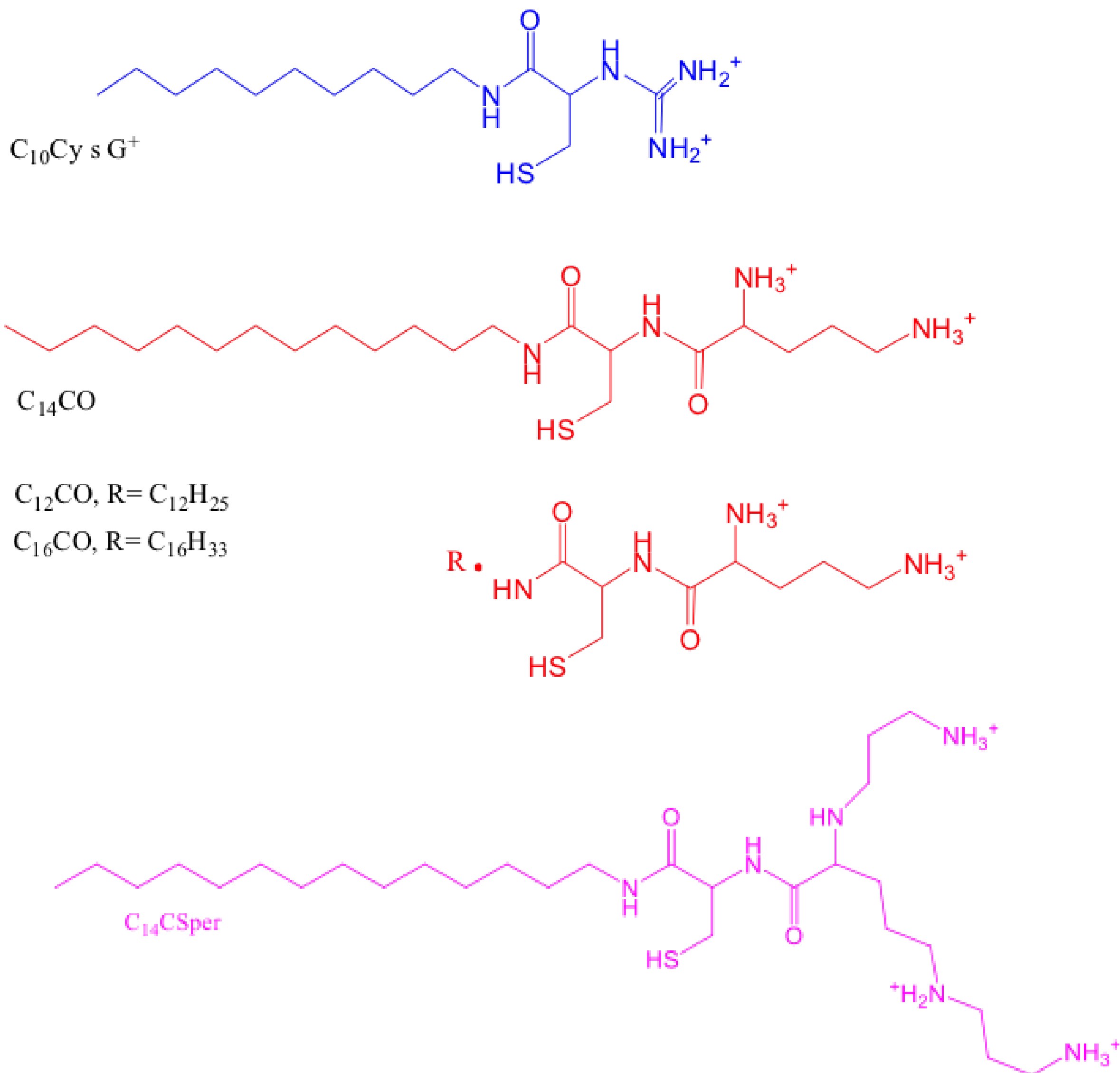

Fig. 3: Structure of some Gemini surfactants

How are Gemini surfactants denoted?

For example, let us consider $C_{10}CysG^+$. The first 'C' denotes carbon and the number following it represents the number of carbon atoms in the acyl chain of the surfactant. The 'Cys' (also sometimes referred as 'C') denotes the cysteine moiety that contains the thiol or sulphydryl group and the last alphabet (in this case G^+) refers to the cationic group. 'G' stands for guanimino, 'O' for ornithine and 'S' for spermine. Thus $C_{16}CS$ refers to a Gemini surfactant with a 16-carbon acyl chain and spermine as the cationic moiety.

As the number of positive charges in the Gemini surfactant increases, its critical aggregation concentration correspondingly decreases. How does one measure the critical aggregation concentration? A commonly employed method is to use a cationic DNA intercalating agent such as YOYO-1 that fluoresces. On complexation with the carrier, the fluorescence is quenched. This quenching can be associated with the critical aggregation concentration.

Another important factor that determines the stability of the Gemini structures in a micellar assembly is the length of the acyl chain. It was discovered that the Gemini surfactant denoted as $C_{10}CysG^{+}$ was very effective in complexing oligonucleotides. However, when anionic or neutral liposomal vesicles were introduced into the system containing the complexes of $C_{10}CysG^{+}$-oligonucleotide, destabilization of the complexes occurred. This may be because of the presence of smaller hydrophobic chainsin $C_{10}CysG^{+}$. If the length of the acyl chain was increased to 14 or higher, the stability of the complexes remained unaltered even in the presence of vesicles. This means that the magnitude of associative forces (mainly hydrophobic) between the neighbouring acyl chains also have a role in stabilizing the oligonucleotide complexes from dissociation.

The morphology of the surfactant-oligonucleotide complexes is generally not exactly spherical. This irregular geometry is most often related to the aggregation of multiple cationic micellar structures with the same oligonucleotide. The wrapping of the oligonucleotide over multiple micelles distorts the spherical geometry. Figure 4 depicts the structure of a typical surfactant-DNA complex.

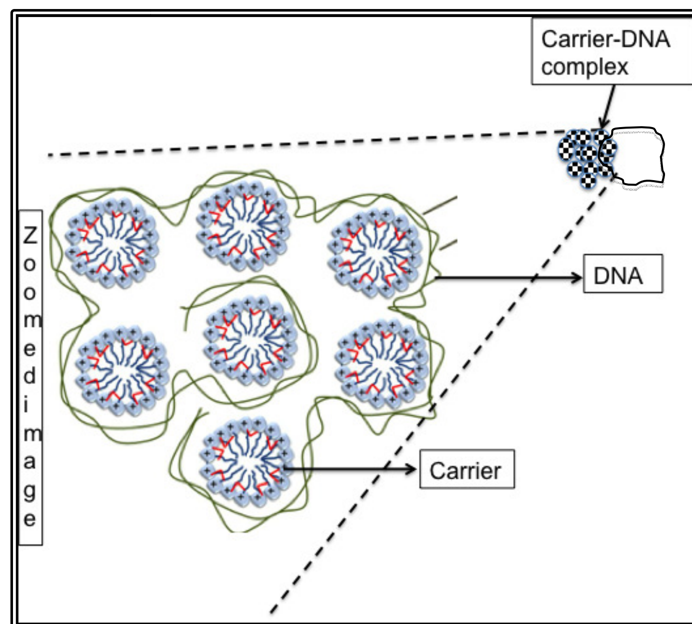

Fig. 4: A typical surfactant (carrier)-DNA complex. The zoomed image shows the presence of micellar structures formed by the surfactant over which the DNA has wrapped itself thus leading to an irregular shape of the complexed structure

This association also has another implication. Not all the oligonucleotide sequences will be present in the interior of the carrier. But it is possible that a part of the sequence might be present in the external surface of the carrier associated through strong electrostatic interactions. This renders the surface negative thereby making it difficult to enter into the cells. Also, if any further modifications are to be carried out in the surface of the carrier for imparting target specificity or to improve its circulation time *in vivo* or to escape from the endosome, the functional groups of the surface may not be available. Hence, another strategy needs to be employed to ensure that the carrier-oligonucleotide surface still remains amenable to modification.

1.2 Surface modification strategy for carrier-oligonucleotide complex

What are the modifications that will be essential? Let us say that we need to deliver the oligonucleotide to a cancer cell. In order to selectively target cancer cells, one needs to link a specific ligand that can bind to the cancer cell surface. One of the commonly over-expressed receptors in cancers, especially those of epithelial origin, is folate receptor. Folic acid is the ligand for this receptor. Hence if one can link the folic acid moiety to the carrier-oligonucleotide complex, it would preferentially bind with cancer cells and will be take up receptor-mediated endocytosis. In order to ensure that the carrier system is not eliminated by the immune system before reaching its destination, it is essential to improve its circulation time *in vivo*. This can be brought about by introducing poly(ethylene glycol) (PEG) chains to the surface of the carrier (Complete discussion on PEGylation and circulation time is given in Module 6).

Now comes the tricky part. As the carrier surface functional groups are not completely accessible for modification with ligands, one has to attempt to link the ligands to the oligonucleotide sequence that is associated with the carrier surface. In the case of a double stranded DNA, there are many options of molecules that can selectively associate with different locations in the DNA. Intercalating agents such as ethidium bromide tend to associate in the interior of the double strand between the nitrogenous bases. Certain sequence-specific proteins exhibit high affinity binding with the DNA. Molecules that have hydrogen bond donor and acceptor properties can associate with the major groove of the DNA even forming Hoogsteen pairing leading to a triple or quadruple helix. *(A Hoogsteen pair refers to the hydrogen-bonded interactions that occur between two nucleotide bases from different strands at the major groove. Such pairs have lead to formation of unnatural triplex and quadruplex structures of DNA).* Similarly, there are certain molecules known as minor groove binders that can bind to the minor groove of the DNA. Peptide nucleic acids (PNA) are a new class of nucleic acid analogues where the ribose sugar is replaced withN(2-aminoethyl)glycine. Thus the backbone of the PNAs

is formed from peptide bonds and the nitrogenous bases (purines and pyrimidines) are linked to the peptide backbone via methylene carbonyl bonds. The PNAs display excellent stability to enzymatic degradation and against denaturation. These PNAs can displace the complementary strand of the DNA and can form Watson-Crick base pairing (A to T and C to G) forming a hybrid structure. Such interactions can also be exploited for anchoring a ligand to the surface of the carrier.

However, there are several limitations that restrict the choice of the DNA associating moiety. In the case of PNAs, their association is sequence-specific and also the cost involved in their synthesis limit their wide-spread use as surface modifiers of carrier-oligonucleotide complexes. Similarly, the DNA-binding proteins also could trigger immune response apart from possessing a prohibitory cost. Intercalating agents and major groove binders may alter the properties and/or influence the structural stability of the DNA. Minor groove binders however, can be used as non-covalent functionalizing agents for DNA. Two of the common minor groove binders are Hoechst 33258 and distamycin. Let us discuss about an example involving these minor grove binders leading to the formation of a chemical moiety incorporating PEG chains and folic acid ligand that can effectively bind to the DNA strand in the surface of the carrier-DNA complex.

Multiple Hoechst and distamycin are covalently linked together using spacer molecules to ensure strong association on the oligonucleotide through multiple anchoring points. Then, PEG chains are linked followed by folic acid. Now, the carrier-oligonucleotide complex can have sufficient circulation time due to PEG chains and can be specifically taken up cancer cells due to the folic acid ligand. Interestingly, the linkers that bind the various components have a disulphide bond. This is important because the presence of the large surface modifiers will increase the size of the carrier-oligonucleotide complex, which can retard its movement especially in the viscous intracellular cytoplasmic milieu. Hence, in order to facilitate the transport of the carrier-oligonucleotide complex within the cell, the surface modifiers should be removed, as their role is primarily to get the complex into the target cell without getting eliminated by the reticuloendothelial system (RES) and they have no further role once the complex is inside the cell. It is here that the disulphide link is very useful. The cell has high levels of reduced glutathione (GSH) as part of its free radical scavenging system. These GSH can effectively reduce the disulphide bonds resulting in its lysis. Consequently, the surface modifiers will be released thus leaving the carrier-oligonucleotide complex free to move about in the cytoplasm. Figure 5 shows the structure of the minor groove binders modified with PEG and folic acid.

This structure when added to the surfactant-DNA complex will immediately bind to the minor grooves at multiple points thereby anchoring the PEG chains and folic acid moieties on the surface of the carrier. Thus effective surface modification of the carrier can be achieved. The only drawback of this strategy is the tedious chemical modification steps involved!

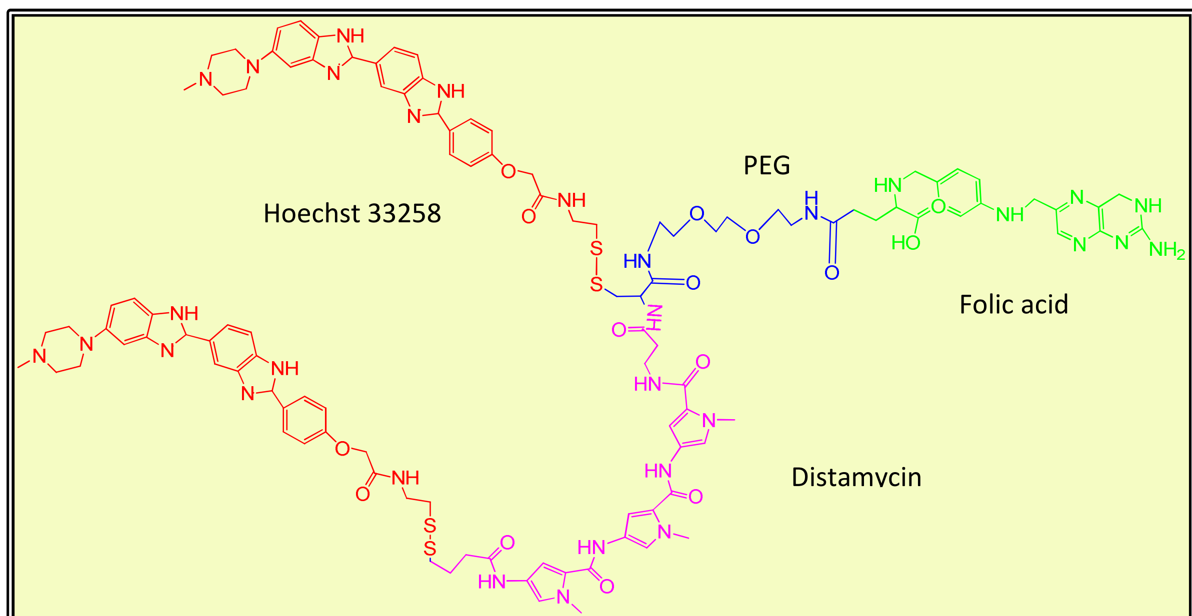

Fig. 5: Structure of a minor groove binding molecule containing PEG and folic acid for long circulation time and specific targeting to cancer cells

2 Virosomes – Transforming toxins to therapeutics!

One of the drawbacks of cationic gene delivery systems such as liposomes is their propensity to be degraded by the lysosomes when they enter the cell through phagocytosis or endocytosis. In either case, the oligonucleotide cargo carried by them is not delivered to the intended target. Hence, to avoid uptake into the endosomes, an attempt was made to introduce the viral fusion proteins in the lipid bilayer. This will enable the liposomal carrier to fuse with the cell membrane and release the contents in the cytosol by-passing the endosomal pathway. These viral fusion protein incorporated liposomes are referred to as '**Virosomes**'. Essentially, a virosome contains the membrane lipids, spike glycoproteins and the fusion proteins of a virus but is devoid of the viral genetic material. The most common viral proteins that have been used to induce membrane fusion are the influenza virus proteins **hemagglutinin** and **neuraminidase**. The major advantage of this strategy is the ability to avoid endosome and directly introduce the contents into the cytoplasm. This strategy helps in achieving efficient delivery without the danger of infections associated with viral delivery systems. Figure 6 depicts the structure of a virosome.

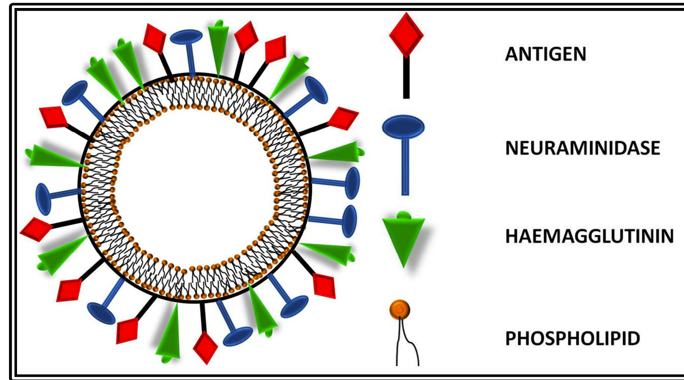

Fig. 6: Schematic representation of a virosome. The antigen molecule is useful if the virosome is used as an adjuvant for vaccine. For delivery applications, the antigen could be substituted with PEG chain or a targeting ligand

Apart from the influenza virus, membrane fusion components from the envelopes of the Semliki forest virus, Sendai virus, vascular stomatitis virus and Sindbis virus have been employed for developing virosomes. However, the presence of viral proteins causes immune response leading to production of antibodies against them. Hence use of these carriers as a gene delivery system is limited. However, the virosomes have found a very effective application due to their ability to stimulate the immune response, especially the T-cells. They have been used as adjuvants for vaccines as well as carriers for peptide and nucleic acid based vaccines. Figure 7 represents the events leading to production of antibodies on introduction of virosomes.

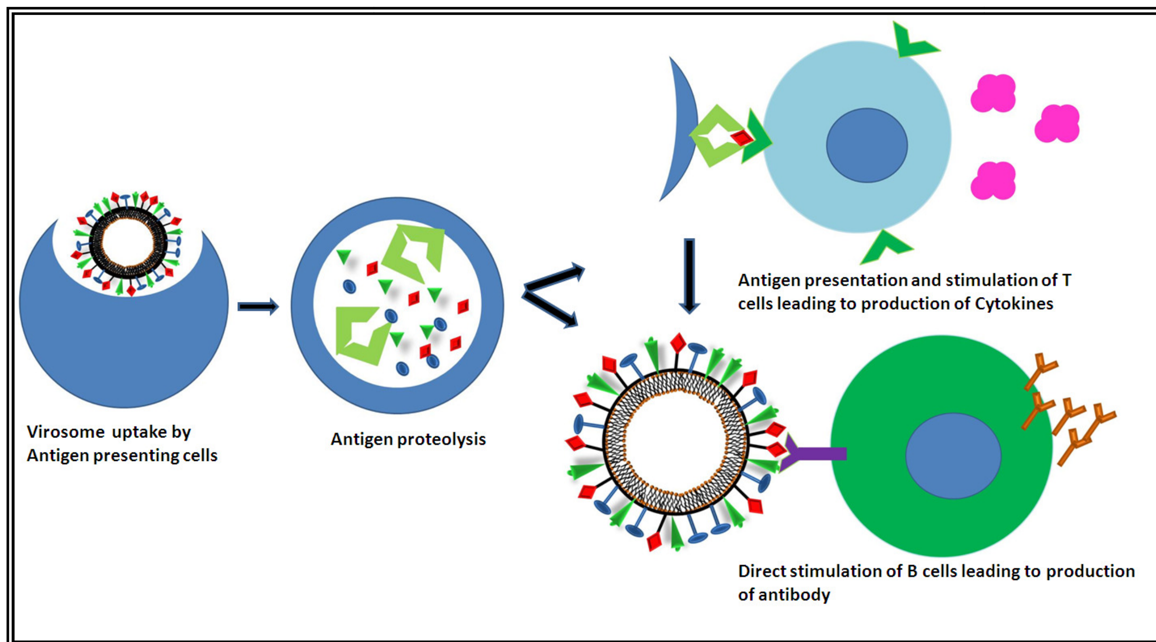

Fig. 7: Use of virosomes as adjuvants for vaccines

A virosome surface modified with an antigen against which antibodies need to be presented. As soon as a virosome is introduced into the biological system, it is recognized by the immune system due to the presence of viral components and is transported into an antigen presenting cell. The virosome is degraded in the antigen presenting cell and the antigen fragment is transported to the surface of the cell and 'presented' for further recognition by the T-lymphocytes. The T-lymphocytes get activated on encountering the antigen and release cytokines leading to the stimulation of the B-lymphocytes which produce specific antibodies against the antigen. The presence of these antibodies provides immunity against any infection resulting from the pathogen (disease causing organism) containing this antigen!

3 Other gene delivery vehicles

The dream of an ideal gene delivery system is yet to be realized as each and every type of carrier has its own merits and demerits. A huge amount of research is directed in designing and developing newer cationic carriers with less cytotoxicity, greater specificity and transfection efficiency. Some of the newer carriers that have evolved include the polysaccharide chitosan, poly(L-lysine),hyperbranchedpolyamidoamine (PAMAM) dendrimers, inorganic gene delivery systems that include modified gold nanoparticles,, magnetic nanoparticles, highly oriented mesoporous silica carriers, calcium phosphates, quantum dots etc. Several approaches have attempted to integrate two different carriers such as liposomes and chitosan or chitosan and PEI etc. These 'hybrid' carriers offer a better combination of properties from both carriers and might represent the future of gene carriers.

4 Reference

Smart Nanoparticles in Nanomedicine (The MML series, Vol. 8), Editors: Reza Arshady& Kenji Kono, Kentus Books, 2006

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