EXOSOME NANOPARTICLE MEDIATED DELIVERY OF siRNA

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILLMENT OF THE REQUIREMENT
FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE
IN
ANIMAL BIOCHEMISTRY

By

SHRUTI SHANDILYA
B.Sc. (Biotechnology)

ANIMAL BIOCHEMISTRY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL-132001 (HARYANA), INDIA

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Approved by :

Rakesh K Tyagi
EXTERNAL EXAMINAR

5/7/17
Dr. DHEER SINGH
MAJOR ADVISOR

MEMBERS, ADVISORY COMMITTEE

Dr. DHEER SINGH
Principal Scientist
Animal Biochemistry Division

Dr. SUNEEL KUMAR ONTERU
Senior Scientist
Animal Biochemistry Division

O. Suneel Kumar

DR. SUNITA MEENA
Scientist
Animal Biochemistry Division

Sunita Meena

Dr. RAJESH BAJAJ
Principal Scientist
Dairy Chemistry Division

Dr. RAJAN SHARMA
Principal Scientist
Dairy Chemistry Division

ANIMAL BIOCHEMISTRY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL -132001 (HARYANA)

Dr. DHEER SINGH
Principal Scientist

CERTIFICATE

This is to certify that the thesis entitled "**EXOSOME NANOPARTICLE MEDIATED DELIVERY OF siRNA"** submitted by **SHRUTI SHANDILYA** for the partial fulfilment of the requirement of the degree of **MASTER OF SCIENCE** in **ANIMAL BIOCHEMISTRY** of the ICAR-National Dairy Research Institute (Deemed University), Karnal (Haryana) is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Date: 05/07/2017

05/07/2017

Dr. DHEER SINGH
MAJOR ADVISOR


Dedicated
to my
Beloved Dada ji,
Family Members
&
Respected guide

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Shruti shandilya

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ABBREVIATIONS

ACRONYMS	MEANING
α	Alpha
β	Beta
#	Number
%	Percentage
µg	Microgram
$\mu g.ml^{-1}$	Microgram per milliliter
µl	Microliter
µM	Micro molar
mg	Milligram
@	At the rate of
<	Less than
>	Greater than
±	Plus minus
≤	Less than equal to
≥	Greater than equal to
°C	Degree Celsius
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
Akt	Protein kinase B
ANOVA	Analysis of Variance
APD	Avalanche photodiode
BACE-1	Beta secretase 1
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CO_2	Carbon dioxide
DEPC	Diethyl pyro carbonate
DOPC	1, 2-dioleoylsn-glycero-3phosphatidylcholine
DOTAP	1, 2-dioleoyl-3 trimethylammonium-propane

DOX	Doxorubucin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
dNTPs	Deoxy nucleoside triphosphates
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme linked immunosorbent assay
EM	Exosome mimic
EV	Extracellular vesicle
Fig	Figure(s)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
g	Gram
Hr	Hour
HER2	Human Epidermal Growth Factor Receptor 2
IL-1β	Interleukin-1 beta
IL-6	Interleukin 6
ILVs	Intraluminal vesicles
IU	International units
Kb	Kilobases
LAMP 2B	Lysosome Associated Membrane Protein 2B
M	Molar
MAPK-1	Mitogen activated protein kinase 1
mA	Milli Ampere
MCC	Manders clocalization coefficient
$mg.ml^{-1}$	Milligram per milliliter
min	Minutes
mM	Mili molar
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger RNA
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide
MVBs	Multivesicular bodies
NaCl	Sodium Chloride
NaOH	Sodium hydroxide

NCBI	National Centre for Biotechnology Information
NDRI	National Dairy Research Institute
ng	Nanogram
NIBS	Non-invasive back scattering
NO	Nitric oxide
PBS	Phosphate buffer saline
PCC	Pearson correlation coefficient
pH	Negative logarithm of hydrogen ion Concentration
PEG	Poly ethylene Glycol
PEI	Poly Ethylene Amine
PTGS	Post transcriptional gene silencing
RAG2	Recombination Activating gene 2
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
RT	Room temperature
Sec	Seconds
SEM	Scanning Electron Microscope
siRNA	Small interfering RNA
TC	Tissue culture
TF	Transcription factor
TLR	Toll-like receptor
Tm	Melting temperature
UV	Ultraviolet

ABSTRACT

Exosomes are tunable nanovesicles of size 30-120 nm in diameter which emerged as a promising drug delivery vehicle for chemotherapeutic drugs, and most importantly for small RNAs i.e. siRNAs and miRNAs. This property of exosomes is due to their natural ability of intercellular cargo transport. A current trend in drug delivery shows the potential use of exosomes as biocompatible, biodegradable and non- immunogenic nanovehicle for delivery of siRNA. However, application of exosomes for therapeutic siRNA delivery may be limited by commonly used loading approaches like electroporation, lipofection and sonication. Lipofectamine is a kind of lipid formulation which can cause potential toxicity to the cells. Recently, electroporation has been employed as a successful loading method for small RNAs. However, this technique induces siRNA aggregation and degradation. Similarly, sonication has been explored as method for loading small RNAs in extracellular vesicle. Therefore, the aim of the present study was to develop an alternative method for loading and delivery of siRNA using exosome. In the present study, we used a chemical conjugate as loading machinery for functionally active small RNAs in milk derived exosomes. A chemical conjugate was prepared and siRNA was linked to it. The present results clearly demonstrated that conjugate mediated loading of siRNA in milk exosomes is found to be efficient as electroporation and lipofection. Further, exosomes loaded with siRNA via chemical conjugate was found to be efficiently taken by Caco2 and HepG2 cells *in vitro.* In conclusion, we demonstrated that chemical conjugate as a natural, scalable, electroporation and lipofection free approach of loading siRNA in milk derived exosomes and subsequently into cancer cell lines for its application as therapeutics.

सारांश

एक्सोसोम्स ऐसे सूक्ष्म पुटिका होती है जिनका व्यास 30-100 nm होता है। ये एक आशाजनक द्वा वितरण वाहन है जिन्हे किमोतेरप्यूटिक द्वा, miRNA और siRNA के लिए प्रयोग कर सकते है। एक्सोसोम्स की यह संपत्ति अंतरिम सामान परिवहन की उनकी प्राकृतिक क्षमता के कारण है। दवाओं की डिलीवरी में एक वर्तमान प्रवृत्ति से पता चलता है कि siRNA की डिलीवरी के लिए बायोकेमपेटि बल, बायोडिग्रेडेबल और गैर इम्युनोजेनिक नैनो वाहन के रूप में एक्सोसोम्स का संभावित उपयोग होता है। हालांकि आमतौर पर इस्तेमाल किया जाने वाला लोडिंग के तरीके जैसे इलेक्ट्रोपोरेशन और लाइपोफेक्षन से एक्सोसोम्स का प्रयोग चिकित्सकीय siRNA डिलीवरी के लिए सीमित हो जाता है। लिपोफेक्टमैन एक तरह का लिपिड सूत्रीकरण का तैयार होना है जो कोशिकाओं को विषाक्तता का कारण बन सकता है। हाल ही में, छोटे RNA के लिए एक सफल लोडिंग विधि के रूप में इलेक्ट्रोपोरेशन को नियोजित किया गया है। हालांकि, यह तकनीक siRNA एकत्रीकरण और गिरावट को प्रेरित करती है। इसी तरह, कोशिकीय पुटिका में छोटे RNA लोड करने के लिए विधि के रूप में सॉनिकेशन का पता लगाया गया है। इसलिए, वर्तमान अध्ययन का उद्देश्य एक्सोसोम्स का उपयोग करके siRNA लोड करने और वितरण करने के लिए एक वैकल्पिक विधि विकसित करना था। वर्तमान अध्ययन में, हमने कृत्रिम रूप से सक्रिय छोटे RNA को दूध से प्राप्त एक्सोसोम्स में डालने के लिए एक रासायनिक संयुग्म का इस्तेमाल एक लोडिंग मशीनरी के रूप में किया गया है। एक रासायनिक संयुग्म तैयार किया गया और siRNA को उसके साथ जोड़ा गया। संयुग्मित एक्सोसोम्स पर बहुक्रियाशील प्रोटीन पर बांध जाता है। वर्तमान परिणाम स्पष्ट रूप से दर्शाते हैं कि दूध के एक्सोसोम्स में siRNA की मध्यस्थता लोड हो रही है, जो इलेक्ट्रोपायरेशन और लिफ़ोफेक्शन के जैसे कुशल साबित हुई है। इसके अलावा, रासायनिक संयुग्तावास के माध्यम से siRNA के साथ भरी हुई एक्ज़ोमोस को Caco2 और HepG2 कोशिकाओं द्वारा कुशलता से लिया गया। निष्कर्ष में, हमने दिखाया कि एक्सोसोम्स में और कैंसर कोशिकाओं में siRNA को लोड करने के लिए रासायनिक संयुग्म प्राकृतिक, स्केलेबल, इलेक्ट्रोपायरेशन और लाइपोफेक्षन से मुक्त एक उपाय है, जिससे इसे चिकित्सा मे प्रयोग कर सकते है ।

CHAPTER –1

Introduction

INTRODUCTION

RNA interference involves a silencing of gene expression through 21-23 nucleotide long short interfering double stranded RNA molecule that directs mRNA degradation in a sequence specific fashion. Small interfering RNAs emerge as a new class of therapeutic modalities as anticancer therapeutics, because of its promising role in post transcriptional knockdown of an oncogene. siRNA therapeutics is now a reality; it has been potentially used to treat the viral infection like HIV, thus involved in inhibition of infection events in HIV-1 cycle (Novina *et al.,* 2002). It has been used as an antiviral agent inhibiting the formation of nucleocapsid protein of Respiratory syncytial virus (De Vincenzo *et al.,* 2010). Advantages of siRNA which makes them assessable to use as therapeutic agent includes safety of siRNA as drug which avoids mutation and teratogenicity risk of gene therapy. siRNA can induce suppression of gene expression in a target cell with only few copies thus showing its high efficacy. Inhibition of gene expression by siRNA requires complementary base pairing with the target gene which determines its property of unrestricted choice of targets. But therapeutic potential of siRNA is hampered by its degradation by extracellular nucleases and rapid clearance from the blood stream. Being hydrophilic it is unable to cross biological membrane and its clinical efficacy is limited by its off target effects. Thus, delivery of siRNA to the tissues and cell of interest requires modifications or formulations. Lipid based nanoparticles, conjugates, polymers and chemical modifications are currently used as delivery vehicle for siRNA which have their own limitations and advantages *in vitro* and *in vivo*. Thus, new biocomapatible, safe, stable, scalable approaches for siRNA delivery need to be developed to enable the therapeutic potential of small RNA.

One such advancement in this field involves the use of exosomes, which turned out to be promising drug delivery vehicle most importantly for siRNAs. Exosomes are biological nanoparticles of size 30-150 nm found in extracellular fluid like saliva, milk, tears. Their natural ability of intercellular cargo transport makes it as a potential drug delivery vehicle. Exosomes have been widely used to deliver chemotherapeutic drug like doxorubicin (Tian *et al*., 2010), Curcumin

(Sun *et al.,* 2010), microRNAs (Valadi *et al.,* 2007). Exosomes loaded with siRNA by lipofection has been used against RAD51 gene in human HeLa and ascites cells (Shatm *et al.,* 2013). Electroporation has been used as successful method for loading small RNAs in exosomes which reduced the expression of BACE 1 mRNA in brain cortex (Alvarez-Erviti *et al.,* 2011). Methods of loading siRNA in exosomes employed till now have certain limitations. Lipofectamine is a chemically synthesized lipoplexes which can cause potential toxicity to exosomes as well as to cells. Whereas electroporation being a mechanical method of loading can induce siRNA aggregation. Therefore, alternative strategy needs to be developed for loading siRNA into exosomes for fulfillment of its safe therapeutic needs. Therefore, the aim of the present study was to develop easy convenient and natural mode of siRNA loading and delivery in cells through exosome with following objectives.

1 Incorporation of siRNA into exosomes

2 Analysis of siRNA delivery using appropriate cells or cell line

CHAPTER –2

Review of Literature

2.1 RNA interference

Small RNA molecules specifically siRNAs and miRNAS inhibits the expression of gene by mRNA degradation in a process known as RNA interference (Kim *et al.,* 2007). In general, this property of siRNA is also referred as *co-suppression*, *post-transcriptional gene silencing* (PTGS), and *quelling*. 2006 Nobel Prize of year 2006 in physiology and medicine was shared by Andrew Fire and Craig C. Mello for their work on RNA interference in the nematode worm *Caenorhabditis elegans* (Fire *et al.,* 1998). RNAi is of key therapeutic advantage because of its ability to knockdown the expression of specific disease causing gene. It is known for its precision, efficiency and stability which are better than antisense technology for gene suppression (Davidson *et al.,* 2011). Hairpin RNA structures are naturally single stranded RNA molecules encoded by genome which gives rise to small RNA molecules i.e. small interfering RNAs and microRNAs by conserved pathways (Svoboda *et al*., 2006). These molecules mediate RNAi either by cleavage of specific sequence by perfect complementary base pairing or by repressing translation and degradation of transcripts for imperfectly matched targets (Kim *et al.,* 2007). Recent advancements in drug development show that gene silencing by RNAi mechanism has great potential (Tiemann *et al*., 2009). Major attributes which makes RNAi to be popular as therapeutics include its remarkable specificity, very low side effects, targeted effects and lastly easy way of their synthesis.

2.2 siRNA

siRNAs are double stranded RNA molecules of 20-23 base pair in length. It is also known as short interfering RNA or silencing RNA (Kawakami *et al.* 2007). Exogenous siRNAs causes transcript cleavage and degradation of their target complementary RNAs in a process of post transcriptional gene silencing. Innate antiviral defence mechanism in nematodes, insects and plants is mediated by processing of viral double stranded RNA into small interfering RNA by Dicer (RNase III enzyme) (Zukerman *et al.,* 2015). The mechanism which involves the degradation of complementary mRNA by siRNA involves the cleavage between the bases 10 and 11 relative to the 5’ of siRNA guide strand.

Activation of siRNA guide strand causes RNA induced silencing complex to undergo multiple round of cleavage which leads to a robust silencing of a target gene (Gavrilov *et al*., 2012). Potential therapeutic applications of RNAi involve mRNA cleavage by PTGS because of its high potency of catalytic gene-silencing pathway and thus siRNA based therapeutics is now a reality (Inoue *et al.,* 2006). In addition, it has become an important tool for validating gene function and drug targeting in post- genomic era (Xu *et al.,* 2015).

2.3 siRNA drug delivery

2.3.1 Chemical modifications of siRNA

Chemical modifications are of great potential and are necessary in cancer therapeutics siRNA delivery system. Chemical modifications can be introduced at the 5'terminus, backbone, sugar or nucleobase of siRNA. The most common modification site of siRNA is the 2'position of the ribose ring, which has been proven to enhance siRNA stability by preventing degradation by endonucleases. The two modification strategies, i.e. 2-O-methyl and 2-deoxy-2-fluoro, are quite well-understood and commercialized, and have been shown to enhance the serum stability of siRNA and increase its in vivo potential (Chiu *et al*., 2004). Some other approaches also exist, such as replacement of the phosphodiester (PO) group with phosphothioate (PS) at the 3'-end of RNA backbone, or the combination of 4-thiolation with 2'-O-alkyl modification (Dande *et al.,* 2006). Through chemical modifications siRNA can acquire advantages such as serum stability, immune escape stability and RNAi machinery access (Braasch *et al.,* 2003). Successful modification involves enhancing serum stability without having negative effects on its gene silencing activity (Layszer *et al.,* 2004).

2.3.2 Lipid mediated delivery of siRNA

2.3.2.1 Lipofectamine: Lipofectamines are cationic liposomes which are widely used for *in vitro* plasmid DNA and siRNA transfection (Santel *et al.,* 2006). Transfection mechanism involves electrostatic interaction between negatively charged nucleic acid and positively charged lipids (Hayes *et al.,* 2006). It is effective siRNA transfection reagent *in vitro* which has improved efficacy of transfection thousand times (Zuhorn *et al.,* 2002).

2.3.2.2 DOTAP & DOPC: Electronegative and neutral liposomes are more biocompatible than cationic liposomes because surface charge of all biological

membranes is negative. DOPC (1,2-dioleoylsn-glycero-3phosphatidylcholine) is a neutral lipid, DOPC encapsulating siRNA liposome was found to be highly effective in reducing expression of Epha2 oncoprotein 48 hours after administration of a single dose in orthotopic model of ovarian cancer (Landen *et al.,* 2005). DOTAP is a cationic lipids (1, 2-dioleoyl-3 trimethylammonium-propane) which form cationic liposomes with negatively charged siRNA, it has been used to deliver siRNA TNF-α in order to suppress lethal reaction to LPS injection in mouse model of sepsis (Sorenson *et al.,* 2003).

2.3.2.3 SNALPS (stable nucleic acid lipid particles): These are lipid nanoparticles approximately 120 nm in diameter, made up of lipid bilayer containing a mixture of cationic and fusogenic lipids coated with diffusible polyethylene glycol (Morrisey *et al.,* 2005). These are highly bioavailable because of its enhanced permeability and retention due to prolonged circulation time in the blood, which leads to its accumulation at site of vascular leakage especially at cancer growth site. SNALP-siPlk1 is found to be successful in 75% reduction in subcutaneous tumor size (Judge *et al.,* 2009). It has been used for the treatment of many diseases including hepatitis B viral infection, dyslipidemia and Ebola (whitehead *et al.,* 2009). To improve SNALP mediated siRNA delivery, lipidoid nanoparticles are developed which are comprised of cholesterol and PEG-modified lipids (Shen *et al.,* 2012). One of the most potential lipidoid drugs was the lipidoid based siRNA formulation 98N12-5, which led to a 75-90% reduction in ApoB or FVII factor expression in hepatocytes in non-human primates and mice (Akinc *et al.,* 2008).

2.3.3 Polymer mediated delivery of siRNA: Polymeric nanoparticles are solid, biodegradable colloidal drug vesicles. These are of two types depending upon the type of material used: water soluble cationic polymers and polymeric nanoparticle. Cyclodextrin is a natural polycationic oligomers (Wang *et al.,* 2009) which was the first targeted siRNA delivery system used for cancer treatment (Davis 2009). In order to improve efficacy of cyclodextrin polymer to deliver siRNA *in vivo* admantane-PEG and admantane-PEG-Transferrin are used (Bellocq *et al.,* 2003, Bartlet *et al.,* 2007). High molecular weight Poly ethylene amine (PEI) has been widely used for nucleic acid delivery under both in vitro and in vivo conditions (Urban-Klein *et al.,* 2005, Grayson *et al.,* 2006). They have high transfection efficiencies but are potentially toxic to cells. Polycaprolactone is

another polymer used for polymeric micelle siRNA drug delivery systems (Sun *et al.,* 2008). Lipid assisted polymeric nanoparticle system are found to be efficient for siRNA encapsulation and delivery, which was fabricated with poly(ethylene glycol)-b-poly(D,L-lactide), siRNA and a cationic lipid, using a double emulsion-solvent evaporation technique (Yang *et al.,* 2011). By incorporation of the cationic lipid, the encapsulation efficiency of siRNA into the nanoparticles was greater than 90%. Systemic delivery of specific siRNA by nanoparticles significantly inhibited luciferase expression in an orthotopicmurine liver cancer model and suppressed tumour growth in a MDA-MB-435s murine xenograft model (Yang *et al.,* 2011). Different delivery methods used upto date are summarised in a table 1.

Table 1: List of important methods used for delivery of siRNA

Method	Nucleic acid delivered	Advantages	Disadvantages
1.Cholesterol	siRNA	Stable and systemic delivery	Non selective delivery
2.SNALP	siRNA	Table and systemic delivery	Non selective delivery
3.Fab	siRNA	Receptor specific delivery	Relitively complex formation
4.Aptamer	siRNA	Receptor specific delivery	Large scale squence screening required
Viral delivery:			
1.Adenovirus	dsDNA	Episomal, no insertional mutagenesis	Immunogenic, dose dependent hepatotoxicity
2.Lentivirus	RNA(shRNA)	Stable expression,transduces non dividing cell	Gene disruption risk,localized delivery
3.AAV	SsDNA/dsDNA(shRNA)	Episomal low genomic integration	Immunogenic,small vector capacity

(Kim *et al.,* 2007)

2.3.4 Conjugates of siRNA

Conjugates are proved as promising system for siRNA delivery and have a potential advantage for cancer therapeutic clinical use because this system of siRNA delivery is simple and well defined. Commonly used conjugates are listed in table 2.

Table 2: Commonly used conjugates for siRNA delivery

Conjugates	Type of conjugation	References
Cholesterol	Binds to 3' terminus of sense strand of siRNA via pyrrolidone linkage	Soutschek *et al.,* 2004
Cell penetrating peptides (CPPS)	Crosslinks with the 3' of the antisense strand of siRNA	Chiu *et al.,* 2004
peptide mimetic of IGF1, D- (Cys-Ser-Lys-Cys)	Conjugated to an amine group of 5' sense strand of siRNA	Cesarone *et al.,* 2007
Antibody	Direct conjugation to siRNA through biotin- streptavidin linkage	Xia *et al.,* 2007
Aptamers-Prostate specific membrane antigen(PSMA)	Direct conjugation to siRNA through biotin- streptavidin linkage	Chu *et al.,* 2006

2.4 Therapeutic advantages of siRNA

2.4.1 High degree of safety: As siRNA does not interact with DNA, risk of mutation and teratogenicity reduces. Thus, it act as safer drug for gene therapy (Xu *et al.,* 2015)

2.4.2 High efficacy: one of the most important properties of siRNA is its high efficacy. Only few copies of it can cause potential suppression of gene expression in a single cancer cell (Larson *et al.,* 2007).

2.4.3 Unrestricted choice of targets: Specificity and unrestricted choice of targets are the key features of therapeutic siRNA. Specificity for a particular target is because of complementary base pairing. Recent developments in molecular biology and whole genome sequencing support this strategy of gene silencing (Jackson *et al.,* 2010).

2.4.4 Comprehensive nucleotide sequence: Databases have been established including human genomic databases, cDNA databases and disease gene databases. The presence of these databases has laid a solid foundation for siRNA drug development (Vaishnaw *et al.,* 2010). Basically exogenous siRNAs are designed against a specific oncogene for the treatment of cancer and can be designed for any disease causing gene according to a particular mRNA sequence (Davis *et al.,* 2010).

2.5 Major limitations of siRNA delivery

Besides advantages and potentials, clinical use of siRNA for cancer therapy is hampered by several barriers. The first biological barrier encountered by administration of siRNA through systemic injection is the nuclease activity in plasma and tissues. siRNA in blood after administration is easily degraded by endogenous nucleases, followed by its filtration in kidneys, uptake by phagocytes and aggregation with serum proteins (Alexis *et al.,* 2008). In serum half-life of unmodified siRNA ranges from several minutes to 1 hour (Layzer *et al.,* 2004). Several studies of bio-distribution of siRNA in animals have shown its highest uptake in kidneys (Van de water *et al.,* 2006). siRNA is negatively charged double stranded hydrophilic small RNA molecules which prevent them to cross a biological membrane. Therefore siRNA need to be packaged in vesicles to facilitate its entry into cells (Xu *et al.,* 2015). Third barrier to siRNA delivery is it's off target effect. Off target effects results in unanticipated phenotypes which further complicates the interpretation of therapeutic benefits of siRNA (Jackson *et al.*, 2010).

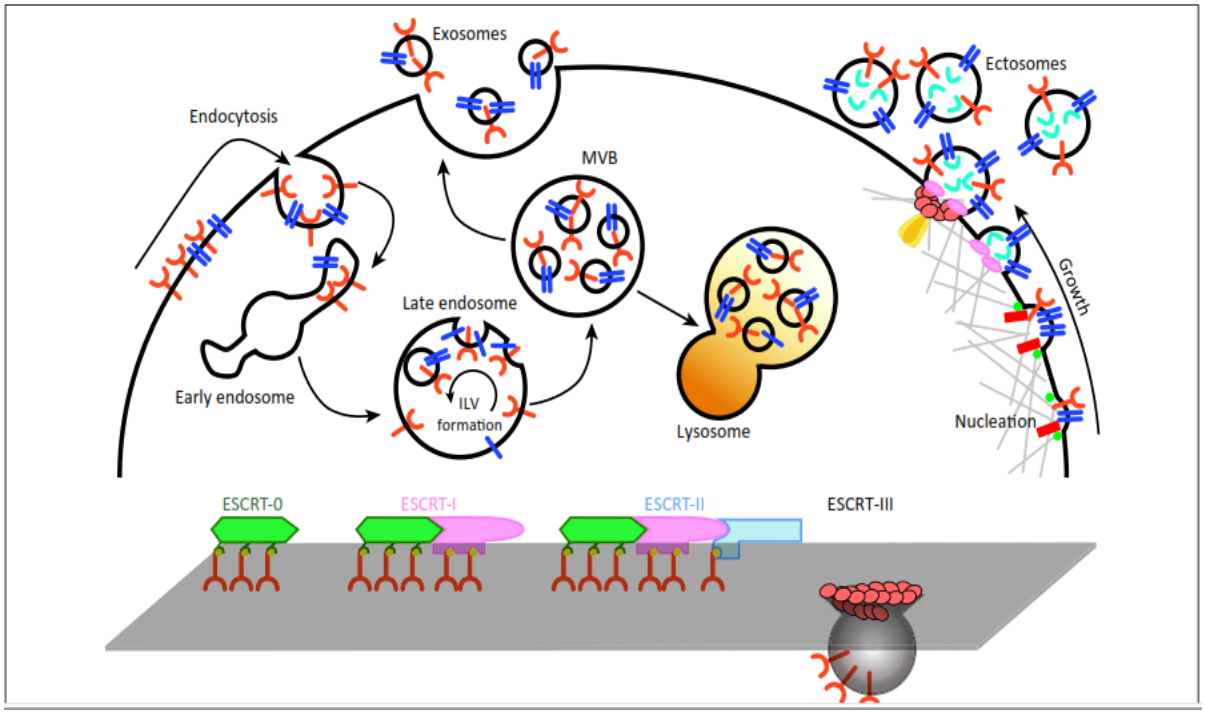
2.6 Hallmarks of good delivery system

In general, for safe and effective delivery of therapeutic drugs in target cells delivery system should be such that it should provide serum stability (Deleavey *et al.,* 2012), Allow immune evasion (Malek *et al.,* 2009), mitigate interactions with serum proteins and non-targeted effects (Wolfrum *et al.,* 2007), resist renal clearance (Jarad *et al*., 2009), enhance vascular permeability to reach tissues (Yu *et al.,* 2009), permit cell entry and endosomal escape to enter RNAi machinery (Whitehead *et al.,* 2009) and ultimately should have low toxicity, which is utmost important part of siRNA delivery (Barquinero *et al.,* 2004).

2.7 Exosomes

Exosomes are the vesicles of endocytic origin of 30-120 nm in diameter which are secreted by variety of cell types (Van *et al*., 2006). Johnstone and colleagues discovered exosomes in 1983. They are synthesized by the inward budding of endosomal membranes and progressively intraluminal vesicle (ILVs) accumulates. This results in the incorporation of trans-membrane proteins into the invaginating membrane and cytosolic components within the ILVs which are secreted as exosomes. Exosome biogenesis is shown in figure 2.1. They contain mRNAs, miRNAs, distinct set of proteins and water insoluble molecules etc. They are involved in cell-cell communication which can take place by three mechanisms. Firstly, membrane proteins of exosomes can interact with the receptors in a target cell and activate intracellular signaling. Secondly, cleaved fragments of the membrane proteins of exosomes can act as a soluble ligand which binds to the target cell surface receptor which can activate the signaling cascade within the target cell. Thirdly, exosomes get fused with the target cell membrane releasing their contents in a non-selective manner which can lead to the activation of signaling (Mathivanan *et al.,* 2010). Because of the nano size (30-150 nm) these natural vesicles are being explored as nanodevices for the development of new therapeutic applications. Exosomes exhibits several attributes as drug delivery vehicle like drug loading capacity, including protein, genetic material, water insoluble molecule, long circulating half-life, membrane penetration capability and intrinsic homing ability (Lai *et al.,* 2010). Drug can be loaded into exosomes either in vivo during biogenesis or in vitro in which isolated

and purified exosomes are loaded with the drug by electroporation, co-incubation etc.



(Cocucci *et al.,* 2015).

Fig 2.1 Diagrammatic representation of exosome biogenesis

2.7.1 Exosome: structure and contents

Exosomes share similar topology to that of plasma membrane (Bellingham *et al.,* 2012). The characteristic surface marker protein of exosomes include heat shock proteins (Hsc 70), lyosomal proteins (Lamp 2b), tetraspanins (CD63, CD81, CD9) and fusion proteins (CD9, flotillin, Annexin) which distinguish them from endogenously derived microvesicles (Johnsen *et al.,* 2014). According to exocarta (Exosome database), exosomes from different cell types and from multiple organisms are known to possess 4563 proteins, 194 lipids, 1639 mRNAs and 764 microRNAs (Frydrychowicz *et al.,* 2015). Carrier protein albumin, GTPases (EEF1A1, EEF2) and cytoskeleton proteins (actin, synenin and myosin) are also present in exosomes (Mittelbrunn *et al.,* 2011). Exosomes also found to contain bioactive lipids such as leukotrienes and prostaglandins (Subra *et al.,* 2010). Varieties of mRNA and microRNAs are present in exosomes which are involved in diverse role such as histone

modification, cell cycle, angiogenesis and migration (Rana *et al.,* 2013, Zoller *et al.,* 2013, Nazarenko *et al.,* 2010, and Skog *et al.,* 2008).

2.7.2 *In vivo and in vitro* drug loading in exosomes

Loading of drugs into exosomes during their biogenesis is *in vivo* drug loading. This method is beneficial for loading transmembrane and cytosolic proteins, higher molecular weight RNA which cannot be loaded *in vitro* (Lai *et al.,* 2013). Different plasma membrane anchors and oligomeric proteins have been loaded into the exosomes (Shen *et al.,* 2011). Loading of drug in purified preparation of exosomes is *in vitro* drug loading. Exosomes efficiently cross the plasma membrane as they are made up of lipid membrane and an aqueous core (Lai *et al.,* 2013). Exosomes act as carrier of hydrophilic and hydrophobic drugs (Yang *et al.,* 2015). Exosomes purified from EL-4 mouse lymphoma cell line can be loaded with curcumin at room temperature (Sun *et al.,* 2010). Different methods adopted for loading of drugs in exosome is summarized in figure 2.2.

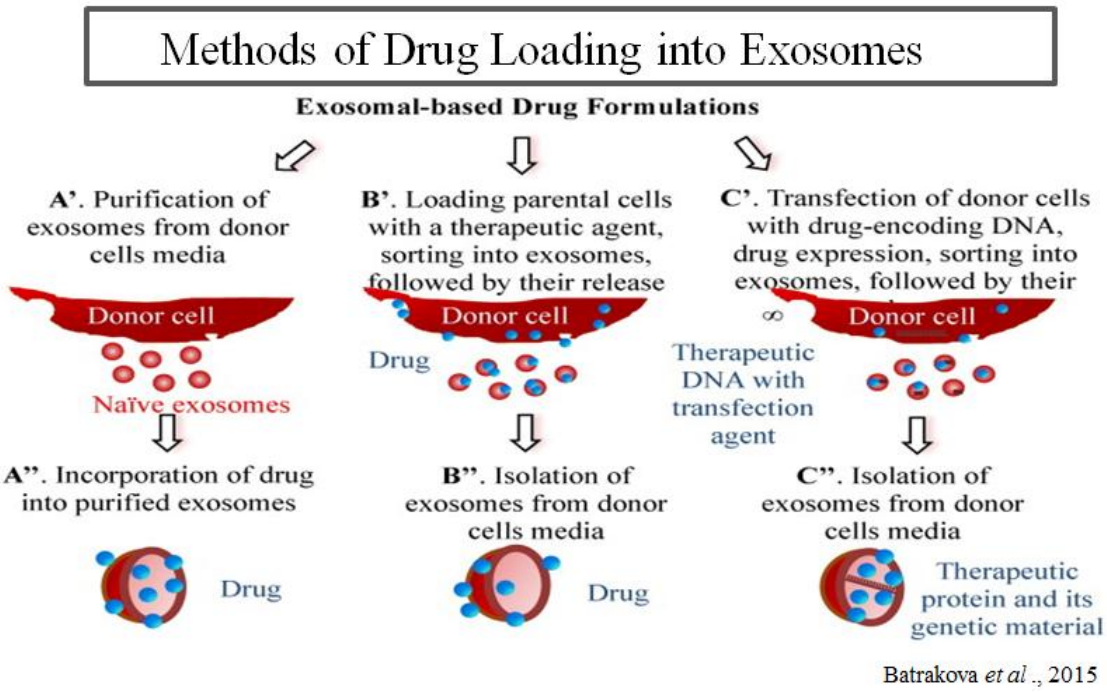


Fig 2.2 Methods adopted for loading of drugs in exosomes

2.8 Comparison of exosomes and synthetic drug carriers

Exosomes present in biological fluids have long half- life which makes them a stable drug delivery vehicle several synthetic delivery vehicles have been used lipid particles and polymeric nanoparticles. Synthetic carriers are sensitive to attack by complement, coagulation factor, opsonins and antibodies in circulation but exosomes are protected when subjected to these factors (Van Den Boorn *et al.,* 2011). Liposomes have been widely used as a drug delivery vehicle but their use is limited as they form aggregates in serum, immunogenic in nature and have limited efficiency *in vivo* (Foged *et al.,* 2012).

2.9 Exosome mediated delivery of therapeutic drugs

2.9.1 Exosome mediated delivery of doxorubicin: Clinical use of Dox is greatly hampered by its dose dependent cardiac toxicity (cardiomyopathy and congestive heart failure). It is the first example of using exosome for targeted delivery of chemoptherapeutic drug Dox to solid tumours in mice. The effectiveness of targeted exosomes encapsulated Dox in improving therapeutic index relative to free Dox at same dose. Systemic administration of this Dox delivery system significantly inhibited tumour growth while causing no overt toxicity (Tian *et al.,* 2014).

2.9.2 Exosome mediated delivery of anti-inflammatory agents: The treatment of inflammatory disorders must rely on targets that are present in both healthy and diseased tissues. Exosomes can deliver anti-inflammatory agents, such as curcumin. Exosome encapsulated curcumin act as a signal transducer and activator of transcription3 (STAT3) inhibitor i.e. JSI124, were delivered noninvasively to microglia cells via an intranasal route (Zhuang *et al.,* 2011). It has also been found that curcumin encapsulated stem cell exosome mitigated T1DM ischemic injury by alleviating synaptic and vascular mitochondrial dysfunction (Kalani *et al.,* 2015). Exosome encapsulated curcumin is found to be more stable and highly concentrated in the blood. Solubility, stability, and bioavailability of curcumin are increased by its incorporation in exosomes (Sun *et al*., 2010).

2.9.3 Exosome mediated delivery of microRNA: Exosomes are natural carrier of microRNAs which implicates its logical therapeutic application in

various disease models (Valadi *et al.,* 2007). Decrease in endothelial cell migration and effector T cells were suppressed by miR150 encapsulated in exosomes (Pan *et al.,* 2011). The level of neurite outgrowth was increased *in vitro* in the presence of miR-133b enriched MSC exosomes as a potential treatment for brain ischemia (Xin *et al.,* 2012). Exosomes could shuttle miR-214 to hepatic stellate cells leading to decreased expression of gene regulating liver fibrosis (Chen *et al.,* 2014; Gressener *et al.,* 2008). High level of miR-146b is expressed in MSC derived exosomes which inhibited tumour growth in xenograft model of GBM (Katakowski *et al.,* 2010). Tumour suppressor microRNAs such as miR-143 and let 7a inhibited the growth of prostate and breast cancer in vivo when delivered through exosomes (Kosaka *et al.,* 2012), no potential toxicity was observed when normal prosthetic epithelial cells were treated with exosome encapsulating miR-143 (Kosaka *et al.,* 2012). miRNA-155 levels in primary mouse hepatocyte and the liver of miRNA-155 knockout mice are significantly increased after injecting exosomes loaded with miRNA-155 mimic. Exosome mediated delivery of miRNA-155 results in functionally more efficient inhibition and less cellular toxicity (Bala *et al.,* 2015). It has also been found that intravenously injected exosomes delivered let-7a miRNA to EGFR-expressing xenograft breast cancer tissue in RAG2–/– mice (Ohno *et al.,* 2013).

2.9.4 Exosome mediated delivery of siRNA: Exosomes are naturally occurring RNA carriers which protects exogenous siRNA from degradation and induction of immune response by it. (Wahlgren *et al.,* 2016). siRNA against *RAD51* and *RAD52* were transfected in exosomes for its safe delivery and effective gene knockdown in a target cell (Shatm *et al.,* 2013. It was found that siRNA delivered through exosomes against *RAD51* was functional and caused the massive cell death of recipient cancer cells (Shatm *et al.,* 2013).

2.10 Methods of loading siRNA in exosomes

2.10.1 Loading via chemical transfection reagent

Chemical treatment (lipofectamine) was used by Shatm *et al.,* 2013 to load siRNA against *RAD51* gene in human HeLa and Ascites cells. For chemical loading of exosomes, siRNA AF-488 was mixed with lipofectamine to form lipoplexes which was then incubated with exosomes for 30 mins at RT, which

effectively down-regulated the target gene expression i.e *RAD51* and *RAD52.* Exogenous siRNAs were successfully loaded into human cell derived exosomes to deliver siRNA to human mononuclear blood cells. Plasma exosomes effectively delivered siRNA to the target cells causing silencing of *MAPK-1* (Wahlgren *et al.,* 2016).

2.10.2 Loading of siRNA via electroporation

Alvarez Erviti *et al.,* 2011 demonstrated the successful use of exosomes for the targeted delivery of siRNA. Dendritic cells were harvested from mice and transfected them to express the neuronal targeting ligand, RVG, coupled to the exosomal membrane protein, Lamp2b. Thus, exosomes secreted from these cells expresses the same protein Lamp2b. After isolation and purification exosome were loaded with siRNA by electroporation against an important protein in Alzheimer pathogenesis (*BACE1*). When exosomes modified with a particular protein were injected intravenously in wild-type mice, a 60% decrease of *BACE1* mRNA in the brain cortex was observed after 3 days. Alvarez-Erviti *et al.,* 2011 manifested the first biotechnological approach for efficient *in vivo* delivery of siRNA by exosome based drug delivery system. Targeted exosomes were generated by El-Andaloussi *et al.,* 2012 through transfection of an expression vector comprising exosomal protein fused with peptide ligand. The targeted exosomes so produced were then loaded with siRNA by electroporation which were utilized for delivery of siRNA *in vitro and in vivo.* The first study on the application of electroporation for the exosome mimic loading of siRNA was given by Yang *et al.,* 2016. Exosome mimics were prepared by serial extrusion of non-tumorigenic MCF 10A cells, which were electrophoresed at different voltages and pulse time in order to optimize siRNA loading. siRNA-EM co-localization percentage was increased from <20% to >80% when voltage was increased from 1100 to 1300 V, indicating a possible voltage threshold for siRNA loading in Ems.

2.10.3 Loading of siRNA in exosomes through sonication

Electroporation has been successfully employed in loading small RNAs in exosomes, but this method of loading induces siRNA aggregation which can limit siRNA functional efficacy. Previously sonication has been employed to generate

chitosan nanoparticles for siRNA delivery (Esmaeilzadeh-Gharehdaghi *et al.,* 2014). Nanosomal formulations were developed and optimized for siRNA delivery to the liver (Kundu *et al.,* 2012). Sonication has been used to load extracellular vesicles with small therapeutic cargoes like paclitaxel to overcome MDR in cancer cells (Kim *et al.,* 2016). Protein cargoes have been encapsulated through sonication in exosomes for Parkinson's disease therapy (Haney *et al.,* 2015). To overcome limitations of electroporation; sonication is emerged as a potential approach for loading small RNAs in EVs. Parameters of sonication and its effect on EV and nucleic acid integrity as well as siRNA aggregation were optimized. Functionality and potency of EVs loaded with siRNA by sonication was assessed by knockdown of the oncogene *HER2,* a therapeutic target that is over expressed in cancers leading to enhanced malignancy (Lamichhane *et al.,* 2016). Methods used for loading siRNA in exosomes are shown in diagrammatic representation shown in Fig 2.3.

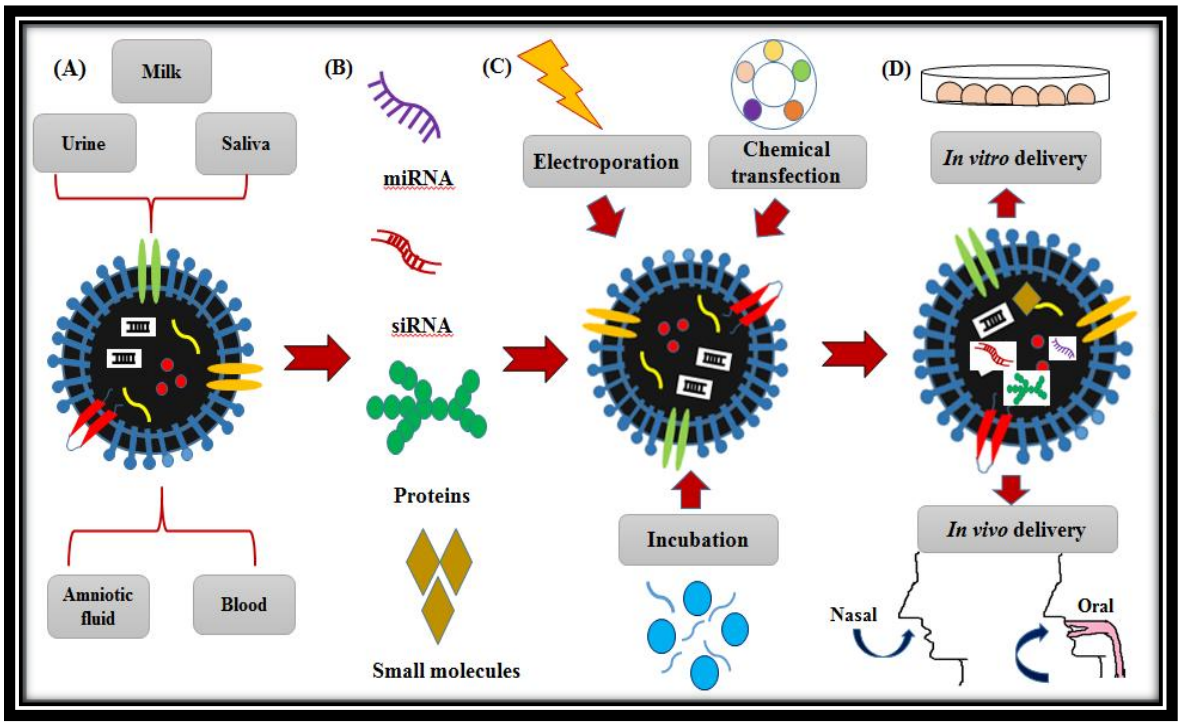


Fig. 2.3 Diagrammatic presentation of different methods used for loading siRNA in exosomes

CHAPTER –3

Materials & Methods

3.1 Objective 1: Incorporation of siRNA in exosomes

3.2 Chemicals/ Equipments

The chemicals and equipments, which are used in the present investigation, have been listed in Appendices and mentioned in the text.

3.3 The procedural steps followed to achieve objective 1 are outlined in Fig. 3.1

3.3.1 Bio-fluid collection and preparation

3.3.1.1 Collection of Cow milk

Cow milk was collected from the healthy, non- pregnant and early lactation Sahiwal cows of National Dairy Research Institute, Karnal. The animals were fed with similar diet and maintained in the same herd. The milk samples were collected in labeled 15 ml tubes at 4˚C.

3.3.1.2 Isolation and purification of exosomes from milk

Milk from Sahiwal cows during early lactation period was obtained from NDRI cattle yard. Exosomes were isolated by precipitation method (exiqon, miRCURY^{TM} exosome isolation kit). Briefly, milk sample was centrifuged at 2000g for 10 mins at 4˚C in order to pellet out cell debris and to remove fat globules. Discarding fat layer supernatant was subsequently collected and centrifuged at 21,500 g for 30 mins at 4˚C to remove casein aggregates and other debris. Discarding fat layer supernatant was collected carefully and centrifuged at 21,500 g at 4˚C for one hour to obtain clear milk whey. Milk whey was collected and filtered through 0.22 µ filter for sterilization. 400µl of precipitation buffer was then added to 1ml of milk whey, mixed properly and incubated for about 3 hours at 4˚C. To precipitate out milk exosomes it was centrifuged at 10000 g for 30 mins at room temperature. Discarding supernatant, exosome pellet obtained was resuspended with 100 µl of resuspension buffer or PBS and stored in aliquots at -20˚C until use.

3.4 Exosomal protein determination

Exosome isolated by precipitation method was quantified by measuring its Protein content. It was estimated in aliquot of exosome preparation using a Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL). Exosomes were usually diluted by 10 fold and were compared in triplicates against serially diluted BSA as a standard as per as the manufacturer's instructions. 10 µl each of blank and diluted exosome were taken in 96 well ELISA plate (Nunc 96 well flat plate). To this 200 µl of reagent-mix (A+B) was added to each of the blank and diluted exosome. Mixture was incubated for 30 mins at 37˚C. A Color change was observed from green to violet in case of test sample showing the presence of proteins. Absorbance was measured at 562 nm using ELISA plate reader (Eon BioTek). A third order polynomial equation with r^2>0.98 for each assay was taken to extrapolate the values from the standard curve.

3.5 Characterization of milk exosomes

3.5.1 Scanning electron microscopy

Scanning electron microscopy was performed using a concentration of ~20 µg/ml. Ten µl of diluted exosomal solution (20 µg/ml) was applied on cover slip and allowed to dry completely. The dried exosomal smear was fixed with 200 µl glutaraldehyde (2%) for 4 hours and then serially dehydrated with 30%, 50%, 60%, 70%, 80%, 90% for 10 mins each respectively. Final dehydration was done with 100% ethanol overnight. The samples were air dried followed by gold coating. Gold coating was done using ion coater (Hitachi IB-3, Japan) with ion current at 6-8 mA and vacuum of 0.05-0.07 torr for 2-4 mins. Scanning electron microscope (EVO®, Carl ZEISS Special Edition-UK) was used to image the coated exosomes.

3.5.2 Dynamic light scattering analysis (DLS)

Size measurements of exosomes were done using Zetasizer (Malvern Instruments Limited, UK). Exosomal solution (10 µg/µl) was diluted in PBS (1:100) and was analyzed in equilibration time of 120 seconds at constant temperature at 25˚C. A laser beam at 632.8 nm was applied to the exosomal suspension and scattered light was detected by an APD (Avalanche Photodiode Detector) at 173˚ and non- invasive back scattering (NIBS) optics. Average of

three measurements was considered to determine the size of exosomes. Size measurements were taken in terms of intensity.

3.5.3 Zeta potential measurements

The zeta potential of exosomes was measured by zetasizer (Malvern instruments ltd, Malvern, Worcestershire, UK). Exosomal solution (10 µg/µl) was diluted in PBS (1:100). DTS 1070 Cell was used for measuring zeta potential. Cell was flushed through with ethanol or methanol to facilitate wetting. Software was set up to use DTS 1070 Cell. Three independent measurements, each composed of 10 runs were carried out at 20˚C. Values of zeta potential was obtained using smoluchowski model and presented as mean ± standard deviation (n=3).

3.6 Loading of exosomes with scrambled AF-488 siRNA

3.6.1 Scrambled siRNA

Allstars Negative Control siRNA, a validated negative control siRNA, was purchased from Qiagen, Hilden, Germany. This siRNA has no homology with any known mammalian gene and is 3' Alexa Flour 488 labeled which ensures its easy assessment by fluorescence microscopy after delivery.

3.6.2 Loading of AF-488 siRNA in milk exosomes using commercially available transfection reagent

In brief, 2.0 µmol/ml of AF-488 siRNA (20 µmol) (Qiagen, Hilden Germany) was mixed with Transfection reagent (X-treme GENE HP DNA Transfection Reagent, Roche) in 100µl of siRNA buffer/ opti-MEM® Reduced serum medium (gibco by life technologies). SiRNA and transfection reagent are taken in 1:3 ratio. Reaction mixture was incubated for 10 mins at room temperature. 300 µl of exosomal solution (10 µg/µl) was added to the reaction mixture of transfection reagent and AF-488 siRNA, which was then incubated for additional 30 mins at room temperature. In order to remove excess of micelles, exosomes were purified using exosome spin columns (MW 3000) (Invitrogen). Encapsulation of AF-488 siRNA in milk exosomes by lipofection is then assessed by fluorescence microscopy as described section 3.8.

3.6.3 Loading of siRNA by electroporation

3.6.3.1 Setting up of electroporation unit

Neon® Transfection Device (Thermo Fisher Scientific) electroporation unit used in the present study was assembled before starting an electroporation. Firstly, it was ensured that Neon® pipette station was connected to the Neon® Device. Neon® tube was then properly fixed in pipette station and was filled with 3.0 ml of electroporation buffer E2 (Neon® 100 µl kit) (Invitrogen by Life technologies). It was ensured that the side electrode of the Neon tube is well connected to the side ball plunger of the Neon® Pipette station. Neon® 100 µl tip was then inserted in a Neon® fixed volume pipette to carry out the electroporation.

3.6.3.2 Procedure for electroporation

Isolated milk exosomes and neon electroporation buffer R was taken in 1:1 ratio. Final concentration of Alexa Fluor 488 siRNA: Milk Exosomes was 500nM: 1 µg.100 µl of electroporation mixture was taken in Neon® gold coated tips for pipette based electroporation. Electroporation was carried out at different voltages, pulse width and pulse time in order to optimize a protocol. Voltage used was 1000 V, 1100 V, 1200 V, 1300 V, 1400 V with pulse width of 10ms and pulse time of 1s. After electroporation, un-encapsulated Alexa fluor-488 siRNA outside milk exosomes was removed by passing a reaction mixture through exosome spin column (Invitrogen, CA, USA). Eluted exosomal suspension was used for further analyses. Encapsulation of AF-488 siRNA in milk exosomes by electroporation was assessed by fluorescence microscopy.

3.6.4 Loading of siRNA in exosomes through chemical conjugation

siRNA was chemically conjugated using a natural macromolecules with EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) based linker. The details of method have not been disclosed as it is yet to be patented.

3.6.4.1 siRNA Loading in milk exosomes via chemically conjugated siRNA

Milk exosomes (200 µg) were incubated with 10 µg of chemically conjugated siRNA complex as developed. Reaction mixture was incubated for specified time and temperature. After incubation, un-encapsulated siRNA from

reaction mixture was subsequently washed by passing through exosome spin column (MW 3000) (Invitrogen, CA, USA). Eluent so obtained was further used for analyses.

3.6.4.2 Preparation of Exosome spin column

Invitrogen Exosome spin columns were purchased from (Thermo Fisher scientific. Spin columns were hydrated with nuclease free water before use as per the manufacturer protocol. In brief, 650 µl of nuclease free water was added to the column to hydrate it for 15-20 mins. After hydration, column was centrifuged at 0.7 g for 2 mins to elute out water from the column. To the obtained gel matrix, 100 µl of reaction mixture was added and centrifuged at 0.7 g for 2 mins at room temperature. The eluted mixture was used for further analyses.

3.7 Fluorescent Labeling of milk exosomes encapsulated with siRNA

Milk Exosomes loaded with AF-488 siRNA after electroporation and Chemical Conjugate mediated was assessed by labeling. Milk exosomes were labeled with red fluorescent dye BODIPY® TR ceramide (Molecular Probes™, Life Technologies) as per manufacturer protocol. 2 µl stock solutions (1 mM in DMSO) of dye were added to 100 µl of exosome preparation to get a working concentration of 20 µM for labeling of exosomes. The exosome-dye mixture was incubated for 20 mins at 37˚C. Unincorporated dye was removed by using exosome spin columns (Invitrogen, CA, USA). The eluted suspension of exosomes so obtained is used for visualization of encapsulated siRNA and further for delivery studies in cultured caco-2 and HepG2 cells.

3.8 Fluorescence Microscopy

Colocalization of milk exosomes and Alexa Fluor-488 siRNA after electroporation and chemical conjugate mediated loading was evaluated by fluorescence microscopy. 2 µl of BODIPY®TR Ceramide (Sigma-Aldrich Co., USA) was added to 100 µl of milk exosome encapsulating AF-488 siRNA after electroporation and Chemical conjugate mediated loading as described above. Labeled Milk exosomes encapsulating siRNA were then visualized using Nikon fluorescence Microscope (Nikon Elipse Ti, Japan). Colocalization percentage was calculated by equation % = AF-488 siRNA containing exosomes/ Total

number of BODIBY® labeled exosomes. Colocalization was further evaluated by calculating colocalization threshold using image J.

3.9 Determination of encapsulation efficiency percentage

Percentage of siRNA encapsulated in milk exosomes was determined by indirect method as described earlier (Papadimitriou *et al*., 2009). Electroporated siRNA loaded exosome and chemically conjugated siRNA exosome complex was centrifuged at 15,000 g for 30 mins at 4˚C. Supernatant was collected in a black well plate (Nunc 96F Microsorp black Microwell) protected from direct contact of light. Fluorescence was measured on Fluorescence plate reader at excitation wavelength of 490 nm and emission wavelength of 525 nm. Encapsulation efficiency was calculated by formula:

EE% = 100-(A2-A1)/ (A4-A3)*100 Where,

A1: Absorbance of blank nanoparticle supernatant

A2: Absorbance of drug loaded nanoparticle supernatant

A3: Absorbance of siRNA solution that has same amount loaded in nanoparticle

A4: Absorbance of vehicle in which siRNA was dissolved

3.10 Determination of colocalization percentage and colocalization threshold

Colocalization percentage was determined by calculating the number of yellow and red pixels using Image J software. Colocalization percentage was determined using a formula: Coloc % = No. of yellow pixels/ No. of red pixels + No. of red pixels *100. Colocalization was determined by Colocalization plugin of Image J software. It calculates Pearson's Correlation coefficient (PCC) (R) and Mander's coefficient (MCC) (M) by costes method (Kenneth *et al*., 2011). PCC ranges from -1 to +1. Value of Mander's coefficient ranges from 1 to zero.

3.11 The procedural steps followed to achieve objective 2 have been outlined in Fig 3.2

3.11.1 Mammalian cell culture

Two cancer cell lines, human adenocarcinoma cell line Caco2 and human liver cancer cell line HepG2 were obtained from National Centre for Cell Science,

Pune, India. These two cell lines were used for the experiment. Basic preparation required for the maintenance of these two cancer cell lines are given below:

3.11.2 Basic preparation in mammalian cell culture

(a) Cleaning of glassware and Sterilization: In general, disposable culture grade plastic wares were used. Reusable glassware was also used after proper cleaning. In brief, all the glassware were washed with neutral detergent, then dipped in chromic acid overnight, followed by thorough rinsing with warm water and finally with distilled water. The glasswares were dried by keeping in oven at 60-70°C wrapped in aluminium foil and stored in dust-free conditions. The glassware to be autoclaved (beakers, bottles etc.) were covered with aluminum foil, tied with a strong thread and autoclaved. Proper sterilization was confirmed by use of an autoclave indicator tape.

(b) Cleaning of laminar air flow chamber, cell culture laboratory, equipments and CO_2 incubator (Thermo Electron Corporation, USA)

Laminar flow hood was cleaned externally with a piece of clean and soft cloth. The work area of laminar flow was wiped with 70% ethanol, using tissue paper. Before starting work, UV lamps inside the laminar flow were switched on for 1 hour. Similarly, after finishing work, laminar flow was cleaned and UV lamps were switched on for another 1 hour. The entire laboratory was first cleaned using a soft cloth. Work surface areas were wiped with 70% ethanol, using tissue paper. Floor was cleaned using an aqueous solution of an appropriate antiseptic agent e.g. Savlon or Dettol. Finally, culture room was fumigated overnight using potassium permanganate and 37% formaldehyde solution. The steel shelves of the incubator were removed carefully and wiped thoroughly with 70% ethanol. The water tray was cleaned thoroughly with warm water and detergent, followed by rinsing with distilled water and dried by wiping with the tissue paper. The tray was filled with autoclaved distilled water. A few crystals of hydrated copper sulphate were added for inhibiting fungal growth. The interior of the incubator was also wiped with 70% ethanol and assembled as before.

3.11.3 Cell culture

Both Human adenocarcinoma cell line,Caco-2 and human liver cancer line, HepG2 cells were grown in Dulbecco's modified Eagle's Medium-high glucose (Sigma-Aldrich Co., USA), supplemented with 10% Fetal Bovine Serum, 2 mM glutamine, Non- essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin and 25 µg/ml of amphotericin. Cell lines were maintained at 37˚C, 5% CO_2 in a humidified chamber. Cells were sub-cultured with a seeding density of 1x 10^6 cells/per flask in T-25 cm^2 flasks. A confluent monolayer was formed in 8-9 days. Caco2 cells between passages 37-55 and hepG2 between passages 35-45 were used for experiments in this study.

3.12. Cytotoxicity Assay

HepG2 cells were seeded at a density of 1 x 10^4 cells/well in 96 well plate at 5% CO_2, 37˚C for 24 hours in a humidified chamber. Cells were washed three times with serum free media and incubated with various concentrations of exosomes loaded with AF-488 siRNA while PBS was used as a control. Four hours post transfection; media was replaced by fresh culture media and incubated for additional 20 hours. Viability of cells was then analyzed by MTT assay per manufacturer's instructions. Briefly, 15 µl of MTT reagent (Sigma-Aldrich Co., USA) was added to each control and exosome treated well. After incubation of cells for 4 hours at 5 % CO_2, 37˚C in a humidified chamber, 150 µl of Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich Co., USA) was added to each well to solubilize formazan crystals followed by spectrophotometric measurement at 580 nm. Cell survival was reported as a percentage of PBS treated control.

3.13 Uptake of siRNA encapsulated exosome *in vitro*

Caco2 and HepG2 cells were seeded at a density of 1x 10^4 cells/well. After 24 hours of culture, media was replaced by fresh serum free and antibiotic free medium. Labeled exosomes encapsulating AF-488 siRNA were incubated with caco-2 and HepG2 cells with a concentration of 250 µg of exosomal protein / 200 µl of media. Delivery of AF-488 siRNA in Caco2 and HepG2 cell lines were assessed by fluorescence microscopy and confocal microscopy. Briefly, exosomes were labeled by method as described above in methods. Labeled exosomes encapsulating AF-488 siRNA were incubated with caco-2 and HepG2

cells with a concentration of 250 µg of exosomal protein / 200 µl of media. Maximum uptake of exosomes occurs in 2 hours as described earlier in our lab (Rani *et al.,* 2017). Two hours post transfection; cells were washed three times with filtered 1X PBS. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich Co., USA) for 20 mins and washed with PBS for three times. Cells were permeabilised by 0.2 % triton X-10 (Sigma-Aldrich Co., USA) for 10 mins followed by washing with 1X PBS three times. Cell nuclei were stained with DAPI solution (Sigma-Aldrich Co., USA) for 5 mins at room temperature. Blue Fluorescence of cell nuclei, red fluorescence of milk exosomes and green fluorescence of AF-488 siRNA were then visualized by fluorescence microscope (Nikon Eclipse Ti, Japan).

3.14 Confocal Microscopy

After incubation of caco-2 and HepG2 cells with BODIPY®TR Ceramide labeled exosomes encapsulating AF-488 siRNA , only labeled milk exosomes and free AF-488 siRNA respectively for 2 hours, cell were washed three times with 1X PBS. Cells were then fixed using 4% paraformaldehyde and permeabilised using 0.2% triton X-100 as described in above method. Cell nuclei were stained with DAPI (Sigma-Aldrich, St.Louis, M.O., USA) for 5 mins at room temperature. Green fluorescence of AF-488 siRNA, red fluorescence of BODIPY and blue fluorescence of DAPI were visualized and recorded on an Olympus FV1000 Filter Confocal Microscope (Olympus Optical CO, Tokyo, Japan).

3.15 *In vitro* Digestion of exosome encapsulating AF-488 siRNA

After loading exosomes with AF-488 siRNA by electroporation and Chemical conjugate mediated loading, exosome encapsulating AF-488 siRNA was digested *in vitro*. Undigested exosome encapsulating AF-488 siRNA was taken as a control. All chemicals and enzymes for *in vitro* digestion were purchased from sigma- Aldrich (Sigma-Aldrich Co., USA) except bile extract which was purchased from Santa Cruz Biotechnology.

3.15.1 Preparation of Digestive Juices

The amount of ingredients used to prepare the respective digestive juices for the digestion of 100 µl of exosome loaded with or without AF-488 siRNA has been given in the table as below:

Salivary Juice	**One digestion**
$CaCl_2(H_2O)_2$	0.25 µl
Mucin II	10 µl
α amylase	0.1 µl
Lysozyme	0.1 µl

Gastric juice	**One digestion**
$CaCl_2(H_2O)_2$	0.075 µl
Mucin II	10 µl
BSA	2.0 µl
Pepsin	2.0 µl

Pancreatic juice	**One digestion**
$CaCl_2(H_2O)_2$	0.075 µl
Mucin II	20 µl
BSA	2.0 µl
Pancreatin	25 µl

Bile juice	**One digestion**
$CaCl_2(H_2O)_2$	0.462 µl
BSA	3.6 µl
Bile	100 µl

3.15.2 *In vitro* digestion

All the digestive juices including saliva, gastric juice, pancreatic juice and bile juice were prepared as described above in 3.15.1. To the 100 µl each of exosome-AF488 siRNA, 133 µl of salivary juice was added and incubated using a rotating wheel for 5 mins at 37˚C followed by incubation with 266.6 µl of gastric juice for 120 mins at 37˚C. Further, reaction mixture was incubated with 266.6 µl of pancreatic juice and 133 µl of bile juice, respectively.

3.15.3 Analyses of loss of AF-488 siRNA encapsulated in milk exosomes after *in vitro* digestion

Loss of exosomes after in vitro digestion was assessed as described earlier (Rani *et al.,* 2017). Briefly, in vitro digested and water control reaction mixture was centrifuged at 12500 g for 15 mins at 4˚C. To the collected supernatant, 400 µl of precipitation buffer (exiqon, miRCURY^{TM} exosome isolation kit) was added followed by incubated for 3 hours at 4˚C.To precipitate out milk exosomes, reaction mixture was centrifuged at 10000 x g for 30 mins at room temperature. Discarding supernatant, exosome pellet obtained was resuspended with 100 µl of resuspension buffer or PBS. Isolated *in vitro* digested exosomes were labeled using BODIPY$^{®}$TR ceramide as described in 3.7 followed by fluorescence microscopy.

3.15.3.1 Measurement of Fluorescence intensity

Exosomes after *in vitro* digestion were isolated by a procedure as described in 3.15.1. 100 µl of siRNA encapsulated exosome isolated after *in vitro* digestion was taken in black well plate followed by Fluorescence intensity measurement**.** *In vitro* digested exosomes encapsulating siRNA were compared to water control (undigested milk exosome encapsulating siRNA). Fluorescence of the respective mixtures was measured using Fluorescence plate reader (Infinite$^{®}$200 PRO, Tecan, Switzerland) at excitation wavelength of 490 nm and emission wavelength of 525 nm.

3.16 Statistical Analyses

Data were analyzed using graph pad prism 5.0 software. Data were presented in the form of mean ± SE (n=3). Stastical analysis was performed using student's t-test and differences with P value less than 0.05 were

designated significant. Localization threshold was determined using Costes test involving analysis of Pearson's and Mander's coefficient.

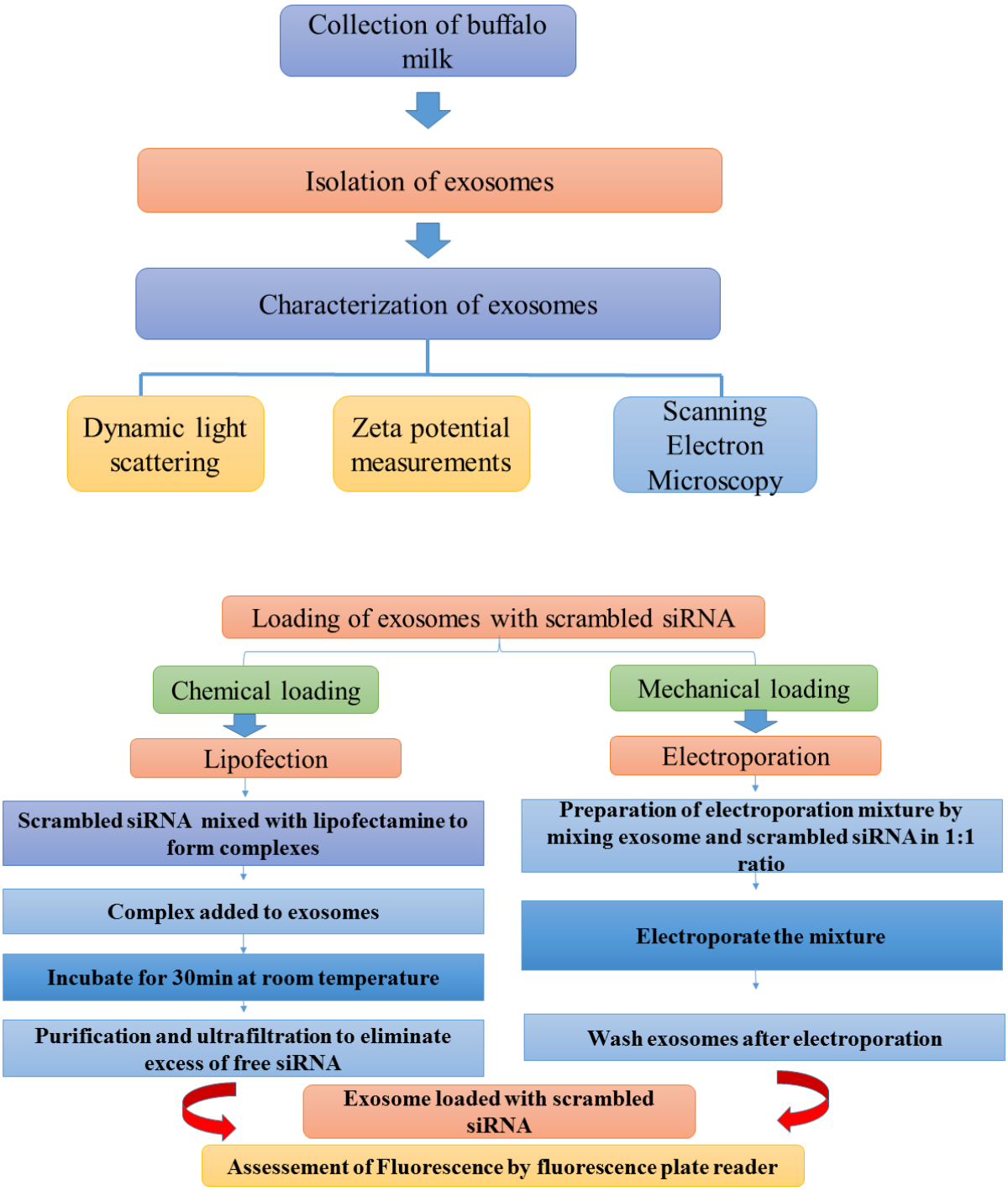


Fig. 3.1 Schematic representation of work plan of objective 1

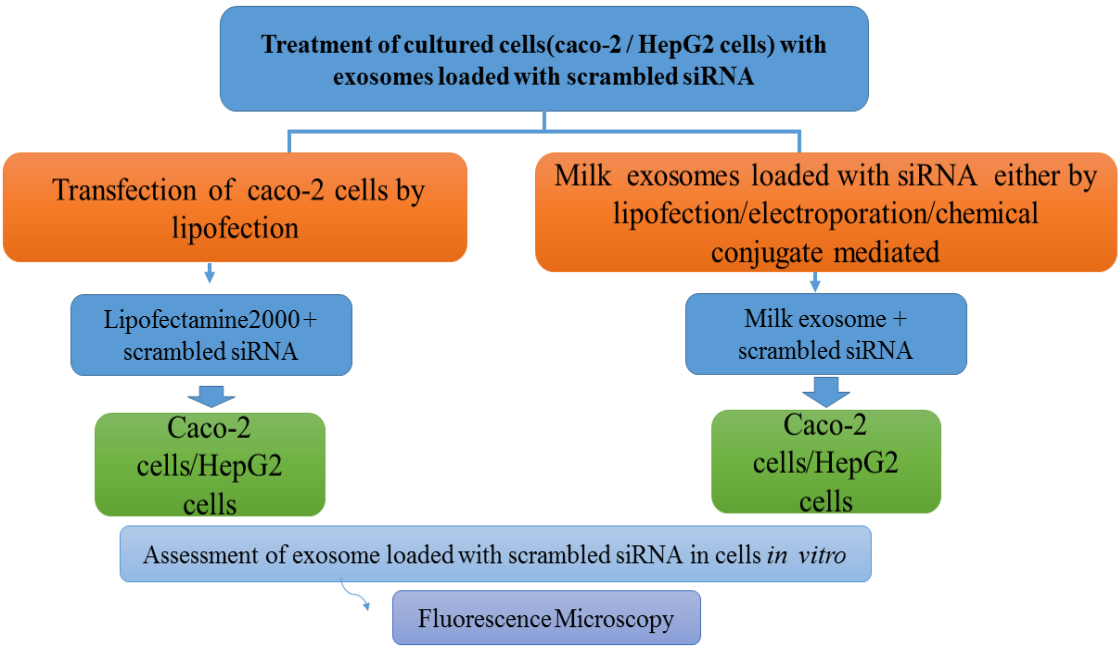


Fig. 3.2 Schematic representation of work plan of objective 2

CHAPTER –4

Results and Discussion

4.1 Objective 1: Incorporation of AF-488 siRNA in exosomes

4.1.1 Exosomal Protein determination

Concentration of Exosomes used in a given experiment was determined in terms of its protein concentration using BCA protein assay kit. Values of exosomal protein concentration were extrapolated through standard curve with r^2= 0.997 plotted using serially diluted BSA standards. Standard curve of BSA has been shown in Fig. 4.1. The protein concentration of exosomes isolated and purified form milk as described in materials and methods was approximately 10 µg/µl (n= 3). The concentration of exosomal protein obtained in our study was found to be as claimed by the earlier studies (Rani *et al.,* 2017) using similar method.

4.1.2 Characterization of milk exosomes

Milk exosomes after being isolated and purified using exosome spin column were characterized by SEM, DLS and Zeta potential measurements. Mean diameter of milk exosomes by number was found to be 120 nm shown in Fig. 4.2 with polydisperity index of 0.3 calculated at 25˚C. Zeta potential measurements show a surface zeta potential of -12 mV with a zeta deviation of -18.3. Zeta potential of -12 indicated that particles are in stable state of dispersion (Kaszuba *et al.,* 2010). The higher the absolute value of zeta potential -25 to +25) greater is the stability of nanoparticle dispersion (Kaszuba *et al.,* 2010). Zeta potential measurements have been shown in Fig. 4.2. These results of zeta potential are consistent with the previous findings obtained in cell derived exosomes having zeta potential of -14.5. Scanning electron microscopy showed the size of exosomes ranging from 30 – 120 nm which supports results of DLS measurement as shown in Fig. 4.2. These results well corroborate with earlier studies where size of milk exosomes has been shown in the rage of 30-150 nm in diameter (Valadi *et al.,* 2007) and PDI of 0.3 indicates that exosomal solution was nearly mono-disperse with no probability of poly-disperity (Malvern instruments ISO). Size and PDI for these nanostructures were in the range as quoted by Rani *et al.,* 2017 and Munagla *et al.,* 2016. Thus, these results

showed that exosome isolated from milk were good enough to fulfill the criteria for proper characterization of exosomes and has been used for the further studies.

4.1.3 Incorporation of AF-488 siRNA in milk exosomes

4.1.3.1 Lipofection based loading ofsiRNA

As described in materials and methods, the method followed by Shatm *et al.,* 2013, was used for siRNA loading in milk exosome using lipofectamine. Lipofectamine 2000 and AF-488 siRNA were taken in 1:3 ratio and incubated with exosomesfollowed by Fluorescence microscopy. All images were taken at a magnification of 40X with the exposure time of 300 ms. Representative fluorescent images has been shown in Fig. 4.3. Fluorescence microscopy images show red pixels representing exosomes and green pixels representing AF-488 siRNA. Merged images of exosomes and AF-488 siRNA were observed as yellow color pixels (red + green = yellow) showing the colocalization of siRNA in the exosomes. It is well established that lipofectamine provides high transfection efficiency and subsequently, high level of gene expression in neurons in *vitro* (Dalby *et al.,* 2004), it can be efficiently used for the loading of siRNA in exosomes. Our results also supported this fact as we also observed good efficiency as compared to electroporation as well as chemically conjugated method (Fig. 4.9). Exosomes are naturally occurring biological carrier of exogenous siRNA. Exosomes isolated from human fibrosarcoma cells (HeLa and HT1080) were loaded with exogenous siRNA by lipofection (Shatm *et al.,* 2013) which were directed against *RAD51* and *RAD52* oncogene.

4.1.3.2 Loading of siRNA by electroporation in milk exosomes

Electroporation conditions were optimized for the efficient loading of AF-488 siRNA in milk derived exosomes. Voltage conditions were standardized with the pulse width of 12 ms and number of pulses being 1. Voltages at which electroporation was carried out was in range of 1000 – 1400 V as shown in the previous studies (Yang *et al.,* 2016). All images were taken at a magnification of 40X with exposure time of 300 ms. Representative fluorescent images taken at different voltages are shown in Fig. 4.4. Merged images contain yellow colored pixels (red + green = yellow) which indicates the colocalization of AF-488 siRNA

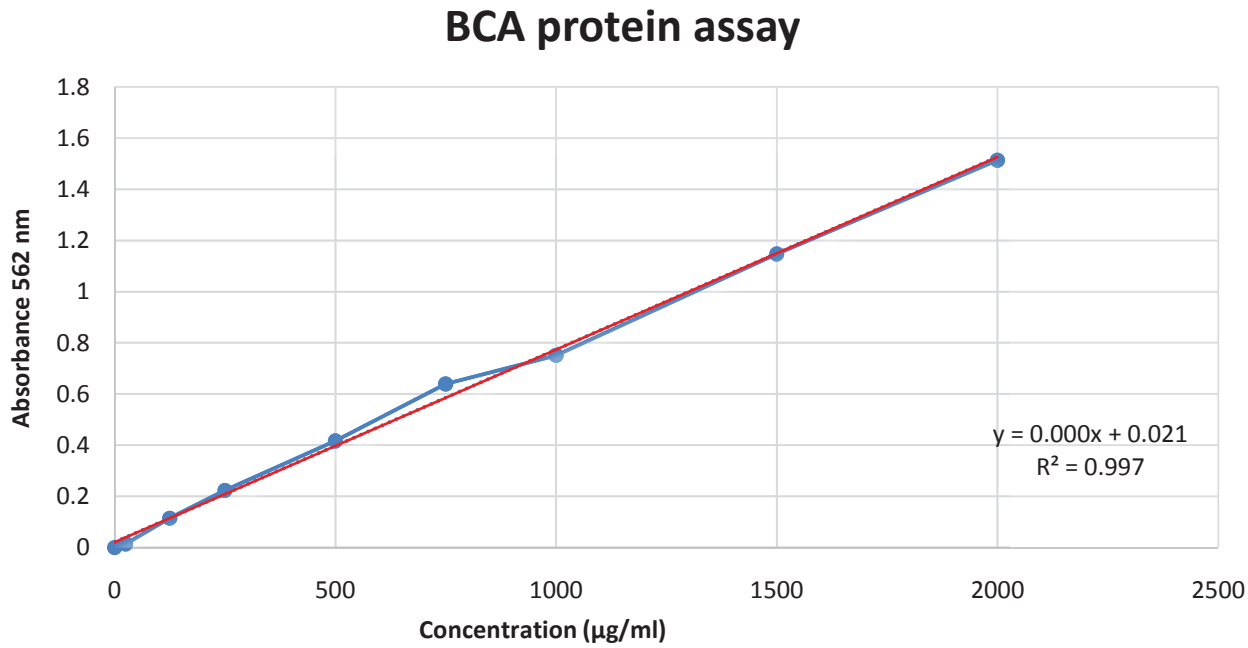
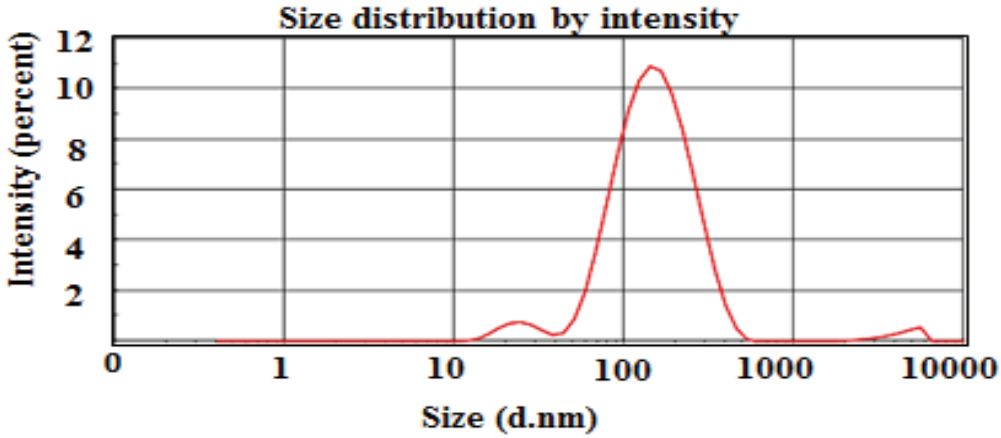
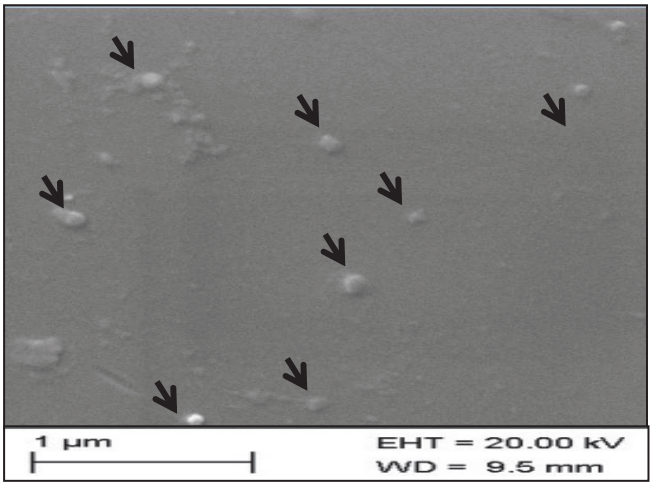


Fig. 4.1 Standard curve of BSA for exosomal protein

(A)



(B)



(C)

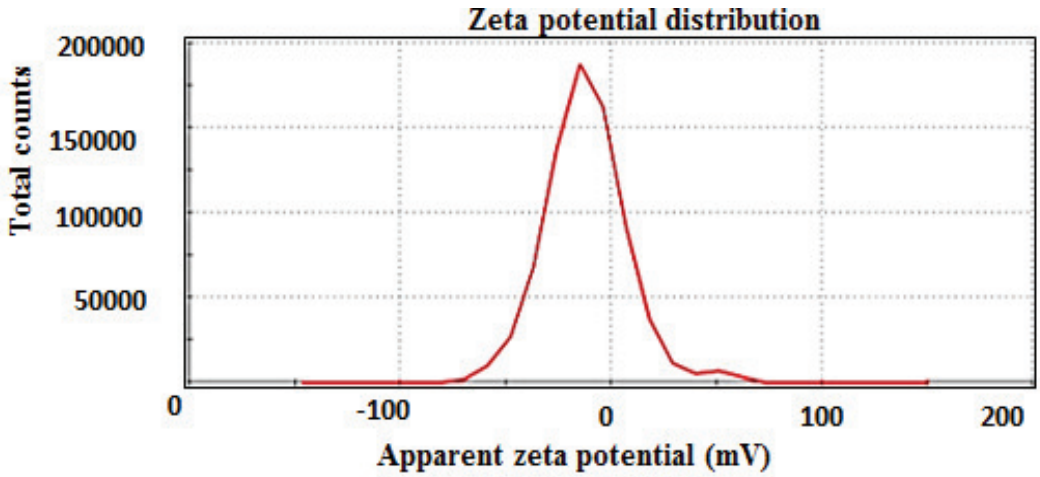


Fig. 4.2 Characterization of milk exosomes. Size distribution analysis of the exosomes using Zetasizer (A) and scanning electron microscopy (B). Analysis of surface zeta potential distribution of milk exosomes using Zetasizer (C).

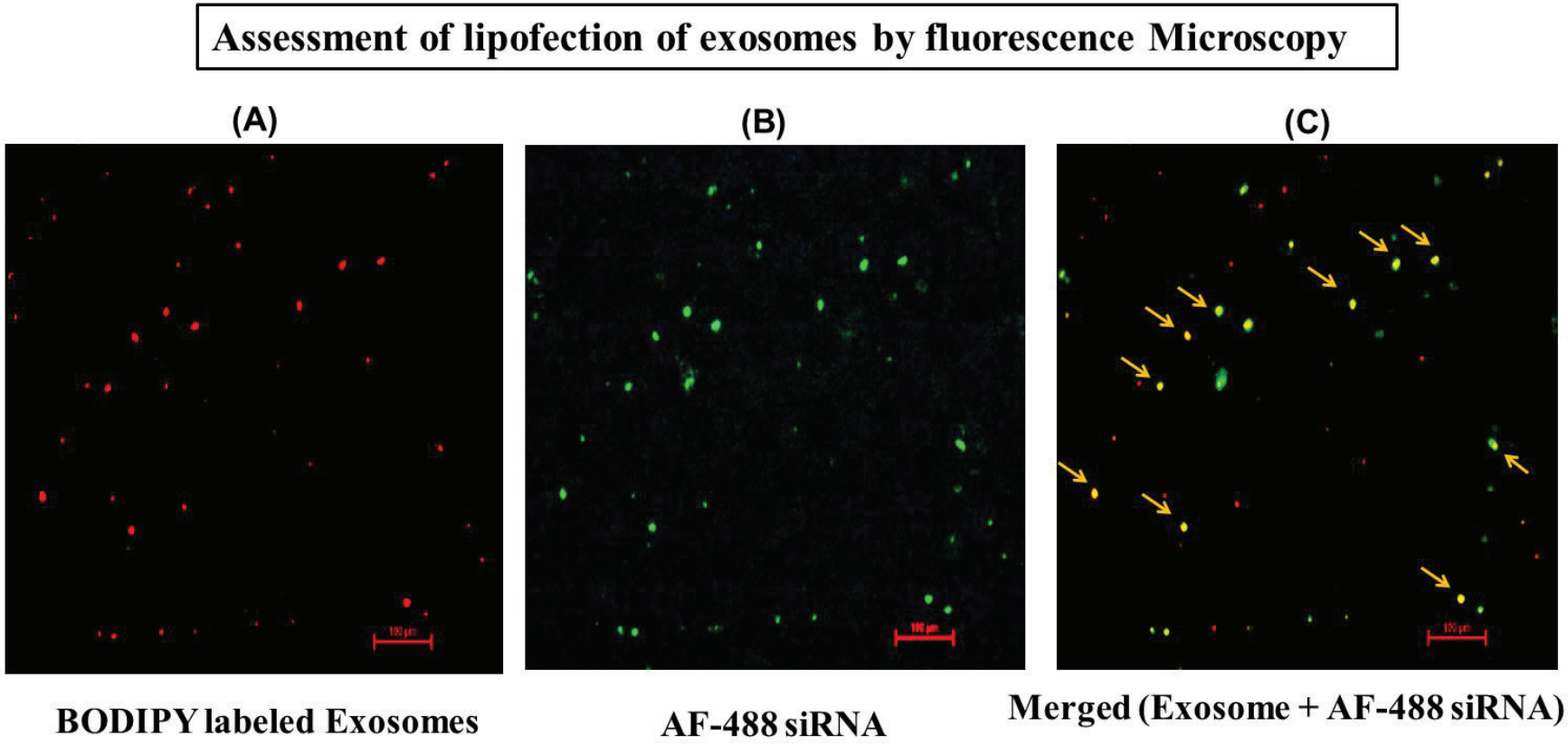


Fig. 4.3 Representative fluorescence microscopy images of milk exosomes after lipofection. (A) BODIPY®TR ceramide labeled milk exosomes (red fluorescence), (B) AF-488 siRNA (green fluorescence) and (C) Merged image representing AF-488 siRNA encapsulated in milk exosomes after lipofection (red + green = Yellow) (n=3).

in exosomes. Similar fluorescence microscopy images of exosomes labelled with PKH dye and cy5-siRNA at different voltages were shown by Yang *et al.,* 2016. Fluorescence microscopy images were used further for determining colocalization percentage and colocalization threshold. Electroporation has been widely employed as a method of loading siRNA in exosomes. RVG targeted exosomes were loaded with GAPDH siRNA by electroporation which were delivered specifically to neurons in the brain resulting in specific gene knockdown of *BACE-1* (Alvarez-Erviti *et al.,* 2011). Similarly, Samir El-Andaloussi *et al.,* 2012 showed the delivery of siRNA *in vitro* and *in vivo* by exosomes loaded by electroporation. siRNA loaded by electroporation in plasma exosomes were efficiently delivered to monocytes and lymphocytes to knockdown the expression of MAPK-1 (Wahlgren *et al.,* 2012). Besides siRNA, miRNA has also been shown to be loaded by electroporation in exosomes derived from T cell and delivered to APCs (Mittelbrunn *et al.,* 2011). Chemotherapeutic drugs like doxorubucin were loaded in exosomes by electroporation to treat cancer (Tian *et al.,* 2014). Our results well corroborate with the previous studies regarding loading efficiency of siRNA in exosome by electroporation.

Colocalization percentage: The co-localization percentage of siRNA encapsulated exosome by electroporation at different voltages has been shown in Table 3. At 1000 V, colocalization percentage was found to be almost 0. At 1100 V, it was increased to 25 ± 5.74 which was further increased at 1200 V to 51.31 ± 5.89. There was sharp increase in colocalization % at 1300 V to 78.31 ± 5.49 followed by its subsequent decrease at 1400 V to 43.63 ± 2.52. Pulse width and pulse voltage was kept constant at 10 ms and 1, respectively with varying voltage during electroporation. It was found that, with the increase in voltage, colocalization percentage increases showing that incorporation of siRNA in milk exosomes was sensitive to voltage. Our results are in resemblance to colcalization percentage of cy5 siRNA in PKH labeled exosome mimics shown by Yang *et al.,* 2016. Accordingly, electroporation at 1300 V, 10 ms and 1 pulse was found to be the best condition to load siRNA in milk exosome.

Encapsulation efficiency percentage: Amount of siRNA encapsulated in milk exosomes after electroporation was determined by indirect method in terms of

encapsulation efficiency percentage (EE %) (Papadimitriou *et al.,* 2009). After loading siRNA by electroporation fluorescence intensity of supernatant was measured giving an approximation of the amount of siRNA loaded in exosomes. Encapsulation efficiency percentage was calculated by formula described in method 3.9. Encapsulation efficiency percentage of AF-488 siRNA in milk exosomes after electroporation was found to be 86.30 ± 2.40, calculated from three independent experiments (n=3). Encapsulation efficiency % so determined was in accordance with siRNA loaded in transferrin lipid nanoparticles (Yang *et al.,* 2014). Encapsulation efficiency of drug praziquantel in PLGA nanoparticle was also calculated by indirect method and was found to be 82 ± 5 % (Mainardes *et al.,* 2005). These results are close to the present study.

Colocalization threshold: Quantitative method of determining correlation between red and green pixels in image is colocalization threshold (Dunn *et al*., 2011). Fluorescence microscopy images taken at different voltages were analyzed for positive correlation between red and green pixels. Threshold for the red and green pixels were set as per Costes *et al*., 2004 and colocalization threshold was determined using colocalization plugin of Image J software. Results of colocalization threshold includes Pearson's correlation coefficient (PCC) for a given image Rtotal, Rcoloc, R<threshold (Pearson 1896). It also includes M1 and M2 which are Mander's colocalization coefficient (MCC) with threshold zero, tM1 and tM2 are MCC with threshold set by Costes *et al* 2004. Colocalized pixel map of fluorescence images at different voltages are shown in Fig. 4.5. PCC (Rtotal, Rcoloc, R< threshold) and MCC parameters (M1 and M2) for the Fluorescence images at different voltages has been summarized in Table 4. +1 PCC value shows a perfect linear correlation, 0 shows no correlation and -1 show a perfect but inverse correlation. Further, for good colocalization high, Rcoloc; good thresholds, R<threshold close to zero; good regression m (slope), b (intercept) and value of M1 and M2 should be close to one. Considering all these parameters, colocalization threshold at voltage 1300 was found to be highest with Rtotal of 0.774, M1 and M2 be 0.731 and 0.829, respectively thus showing a high positive uphill correlation between red and green pixels. Results (Table 4) of present study showed that Rtotal was found to be increased from zero at 1000 V, to 0.518 for 1100 V and 0.55 for 1200 V showing a moderate

Voltage	colocalocalization percentage
1000V	0.03 ± 0.01
1100V	25.00 ± 5.74
1200V	51.31 ± 5.89
1300V	78.31 ± 5.49
1400V	43.63 ± 2.52

Table 3. Tabular representation of colocalization percentage after electroporation at different voltages (1000-1400 V) with a pulse width of 10 ms and number of pulse being 1 (n=3).

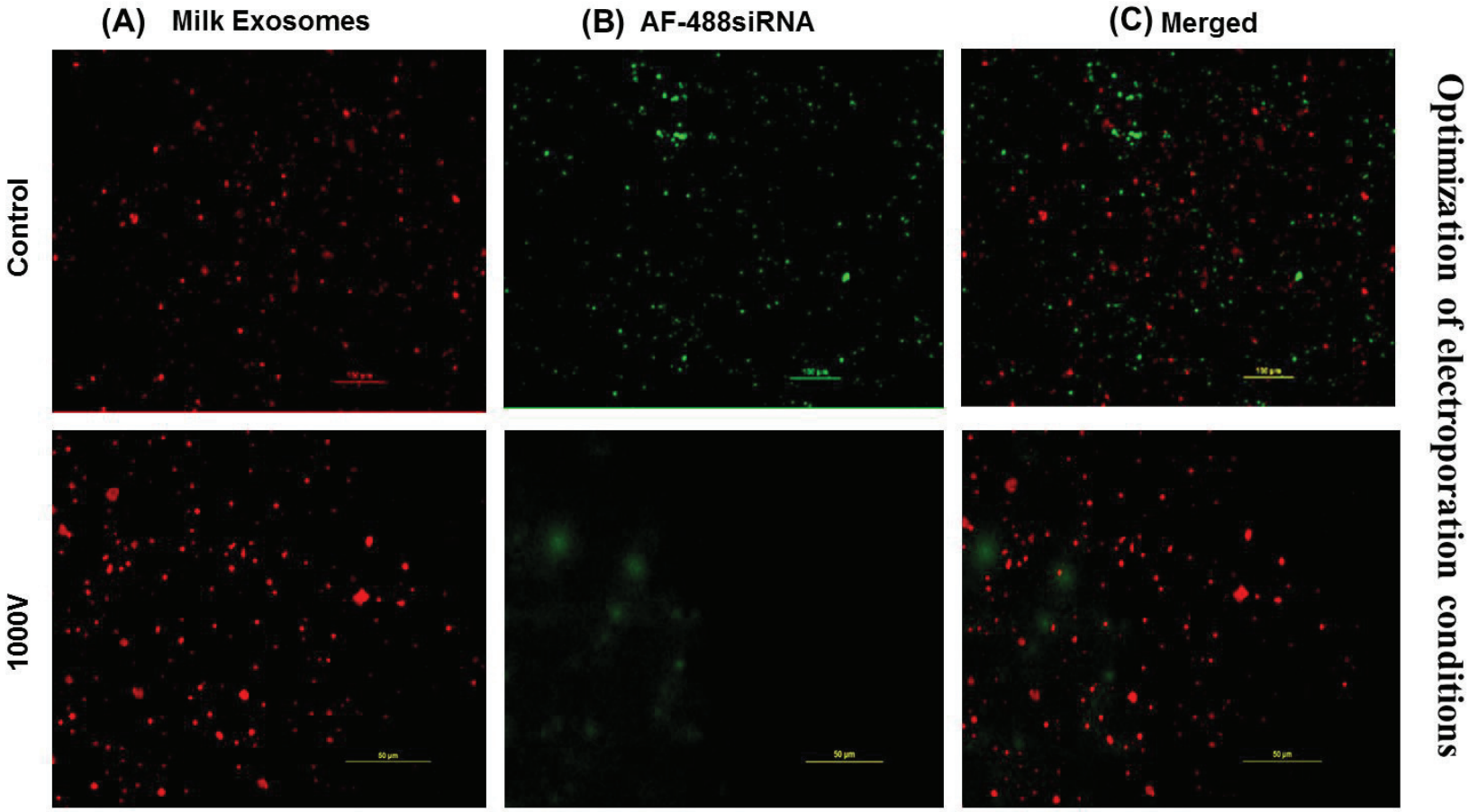


Fig. 4.4 Representative fluorescence microscopy images of milk exosomes and AF-488 siRNA after electroporation at 1000 V and of control (mixture of exosome +AF-488 siRNA without electroporation) (A) BODIPY®TR ceramide labeled milk exosomes (red fluorescence), (B) AF-488 siRNA (green fluorescence) and (C) Merged image of milk exosome and AF-488 siRNA (red + green = yellow) of control and at 1000 V, respectively (n=3).

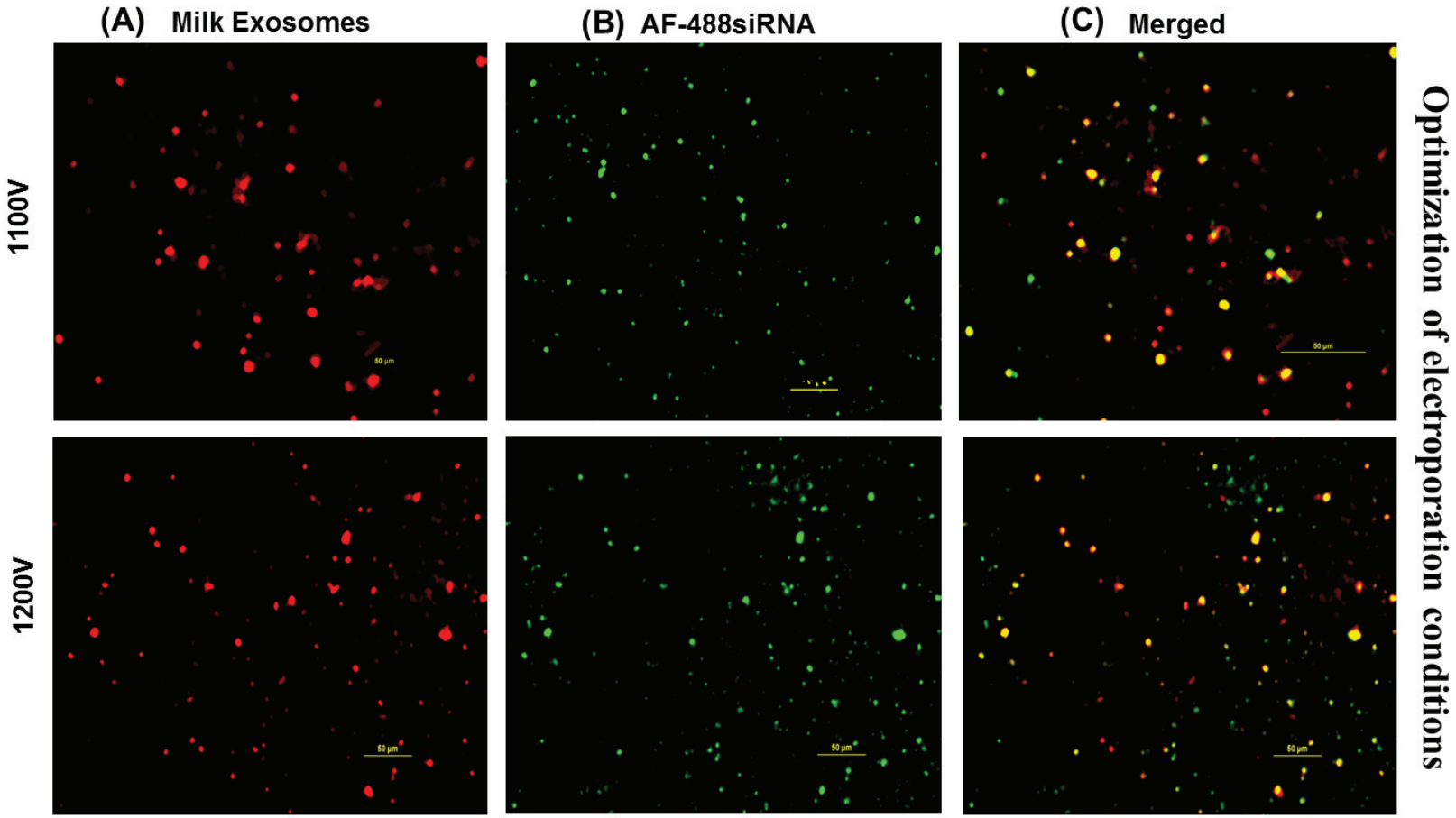


Fig. 4.4 Representative fluorescence microscopy images of milk exosomes and AF-488 siRNA after electroporation at 1100 V and 1200 V (A) BODIPY®TR ceramide labeled milk exosomes (red fluorescence), (B) AF-488 siRNA (green fluorescence) and (C) Merged image representing AF-488 siRNA encapsulated in milk exosomes after electroporation (red + green = yellow) at 1100 V and 1200 V, respectively (n=3).

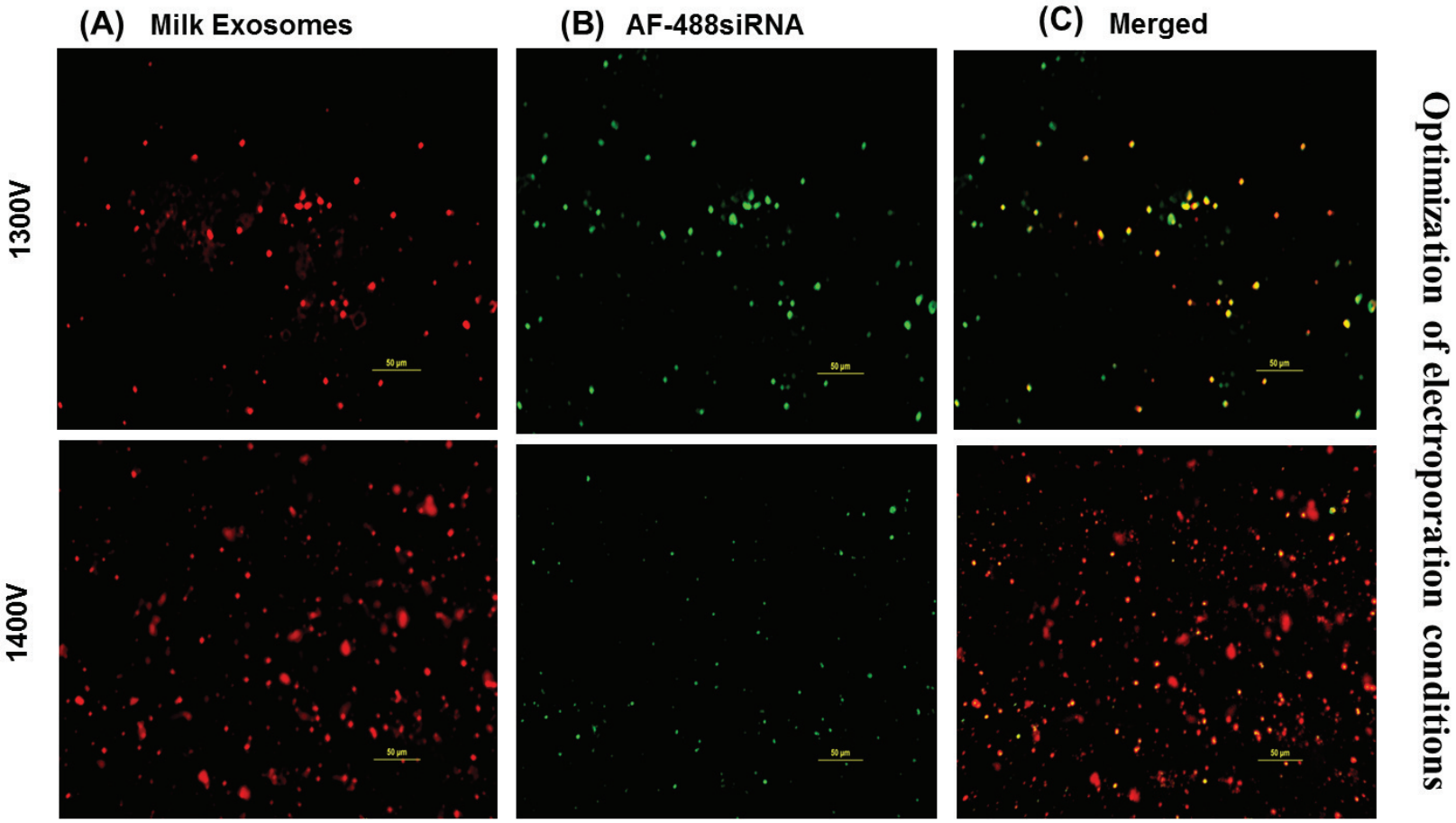


Fig. 4.4 Representative fluorescence microscopy images of milk exosomes and AF-488 siRNA after electroporation at 1300 V and 1400 V (A) BODIPY®TR ceramide labeled milk exosomes (red fluorescence), (B) AF-488 siRNA (green fluorescence) and (C) Merged image representing AF-488 siRNA encapsulated in milk exosomes after electroporation (red + green = yellow) at 1300 V and 1400 V, respectively (n=3).

uphill linear relationship. Rtotal was decreased further to 0.315 with Rcoloc of zero at 1400 V showing a poor uphill correlation.Colocalization threshold showed the trend similar to that of colocalization percentage i.e. it was found to be increased with the increase in voltage. Considering these results, fluorescence image at 1300 V was found to be the highest while Rtotal, Rcoloc, and R<threshold was almost zero, thus it was chosen as an optimized voltage for electroporation. This is similar to colocalization of fluorescently labeled transferrin in MDCK cells showed PCC value of 0.69 representing a perfect colocalization (Dunn *et al.,* 2011) and MCC of transferrin in MDCK cells was close to 1 which showed the perfect colocalization of one probe with another probe (Manders *et al.,* 1993). This is in accordance with our results obtained for colocalization of AF-488 siRNA with exosomes at 1300 V. This trend of PCC and MCC values were also observed for the fraction of one fluorescently labeled protein that colocalizes with another fluorescently labeled protein (Costes *et al.,* 2004).

4.1.3.3 Loading of AF-488 siRNA via chemical conjugate in milk exosomes

As anelectroporation and lipofection free approach, AF-488 siRNA was loaded in milk exosomes with the help of novel method using chemical conjugate. As indicated in materials and methods, we did not disclose the details of method as method is yet to be patented. However, in principle, chemical conjugate interacts with milk exosomes and AF-488 siRNA was subsequently loaded in milk exosomes. Loading of AF-488 siRNA was assessed by fluorescence microscopy, in which BODIPY ®TR ceramide emits red fluorescence and AF-488 siRNA emits green fluorescence. Yellow color pixels (red + green) confirm the presence of AF-488 siRNA in milk exosomes.Similar, fluorescence microscopy images were observed in previous studies of PKH labeled exosomes of MCF10A cells loaded with cy5 siRNA (Yang *et al.,* 2016). All images were taken at 40X with exposure time of 300 ms. Representative fluorescence images of exosomes and AF-488 siRNA has been shown in Fig. 4.6. To the best of our knowledge there are no reports regarding chemical conjugate mediated loading of AF-488 siRNA in milk exosomes.

Colocalization percentage: Chemical conjugate mediated loading of AF-488 siRNA in milk exosomes was confirmed by determining the percentage

colocalization of green over red pixels as described above in detail (Lagache *et al.,* 2015). Colocalization percentage in chemical conjugate mediatedloading was found to be 41.7± 2.39.

Encapsulation efficiency percentage: Encapsulation efficiency percentage of AF-488 siRNA in milk exosomes after ligand mediated loading was found to be 88.03 ± 7.80, calculated from three independent experiments (n=3).

Colocalization threshold: The results showed Rtotal of 0.791, Rcoloc of 0.482, R< Threshold is equal to zero; M1 and M2 are 0.63 and 0.98, respectively. These results showed a high uphill positive correlation for the colocalization of green pixels over red pixels. This shows that a chemical conjugate efficiently loaded AF-488 siRNA in milk derived exosomes.

4.1.4 Comparison of electroporation versus conjugate mediated siRNA loading

Encapsulation efficiency, colocalization percentage, and colocalization threshold for the exosomes loaded by electroporation and by chemical conjugate were compared as shown in Fig. 4.7**,** Fig. 4.8, and Table 4, respectively. Since, lipofection is commonly used and standardized method of loading siRNA in exosomes therefore, it was not compared with electroporation and chemical conjugate mediated loading. Results showed that that there was no significant difference (P>0.05) in encapsulation efficiency between novel chemical conjugate mediated loading (88.03 ± 7.80) and well established electroporation (86.30 ± 2.4.) based siRNA loading in milk exosome. Similar results were also obtained when intensity based quantification was done by calculating colocalization percentage. Colocalization percentage was found to 51.31 % in electroporation at 1200 V while in case of chemical conjugate mediated loading of milk exosomes was 41.7%. Further, to confirm whether chemical conjugate mediated loading of siRNA in milk exosomes was efficiently comparable to that of electroporation, colocalization threshold was compared. There was no significant difference between PCC and MCC for chemically conjugated mediated loading (Rtotal-0.791, Rcoloc-0.482, R<threshold-0.001, M1-0.630, M2-0.985) and electroporation (Rtotal-0.774, Rcoloc-0.482, R<threshold-0.001, M1-0.731, M2-0.829). Comparison of fluorescence microscopy images of

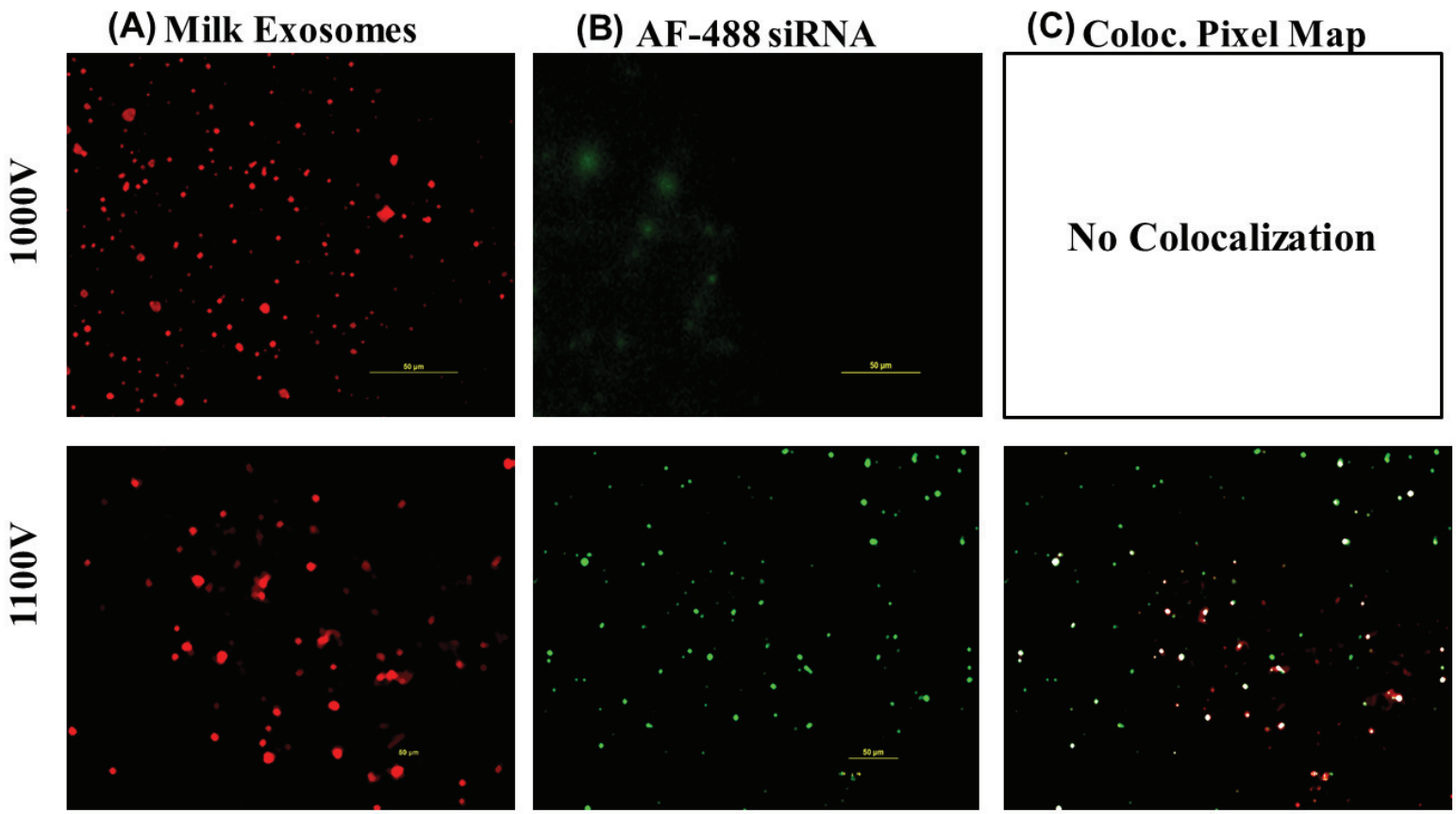


Fig. 4.5 Colocalized pixel map showing the correlation between red pixels (milk exosomes) and green pixels (AF-488 siRNA) (A) BODIPY®TR ceramide labeled milk exosomes (red fluorescence), (B) AF-488 siRNA (green fluorescence) and (C) Colocalized pixel map of exosomes after electroporation at 1000 V and 1100 V, respectively (n=3).

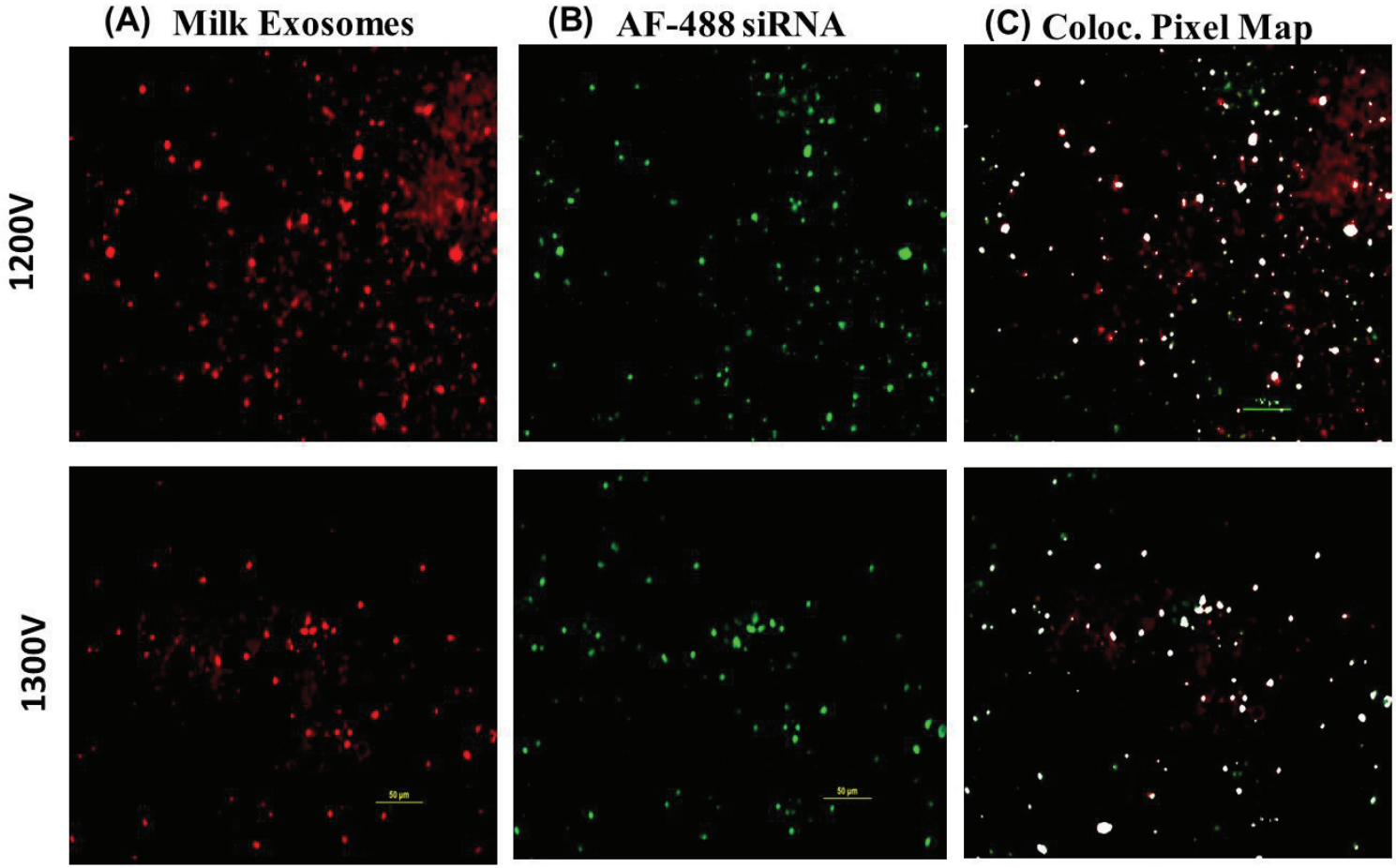


Fig. 4.5 Colocalized pixel map showing the correlation between red pixels (milk exosomes) and green pixels (AF-488 siRNA) (A) BODIPY®TR ceramide labeled milk exosomes (red fluorescence), (B) AF-488 siRNA (green fluorescence) and (C) Colocalized pixel map of exosomes after electroporation at 1200 V and 1300 V, respectively (n=3).

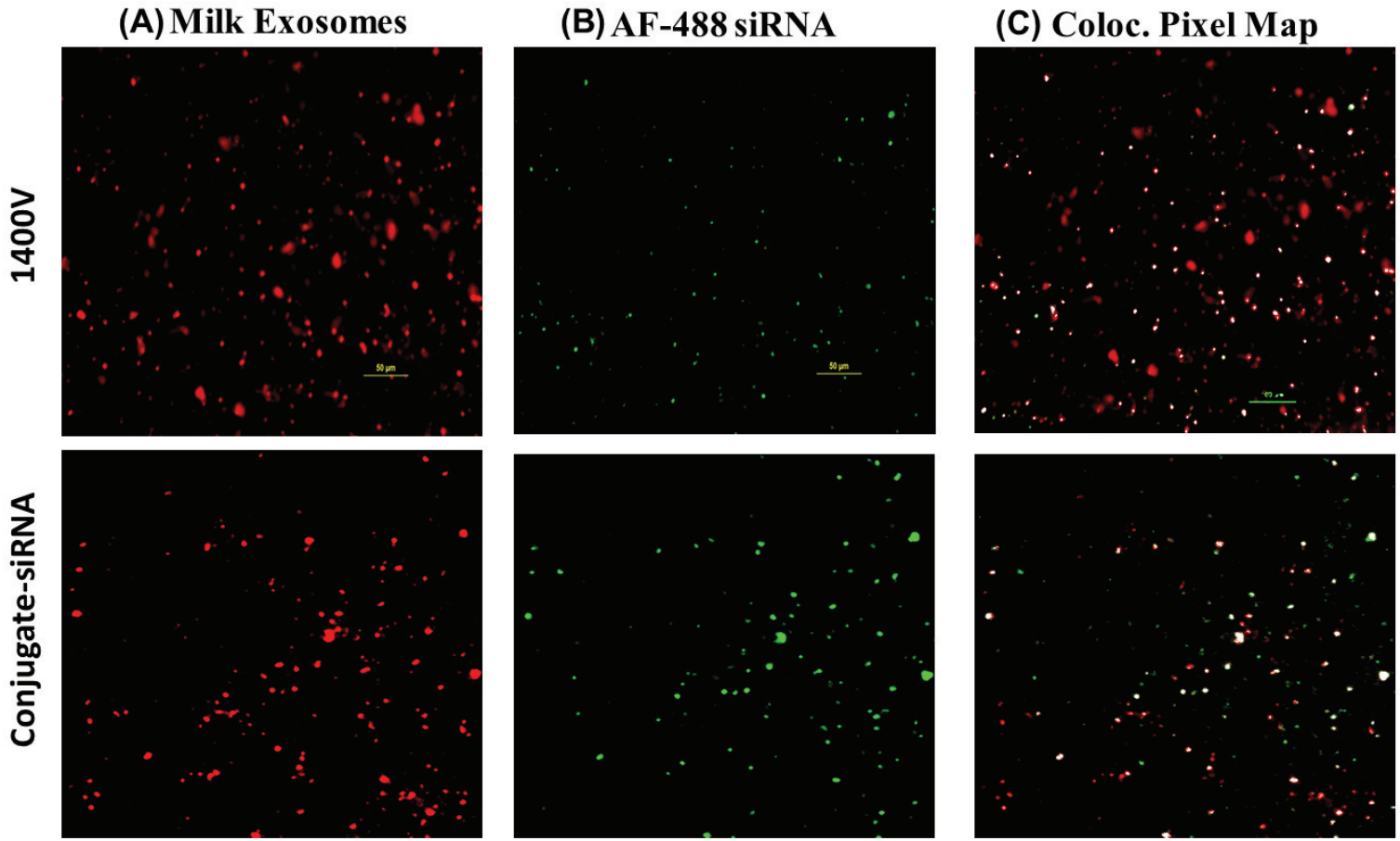


Fig. 4.5 Colocalized pixel map showing the correlation between red pixels (milk exosomes) and green pixel (AF-488 siRNA) (A) BODIPY®TR ceramide labeled milk exosomes (red fluorescence), (B) AF-488 siRNA (green fluorescence) and (C) Colocalized pixel map of exosomes after electroporation at 1400 V and of conjugate mediated loading of exosomes, respectively (n=3).

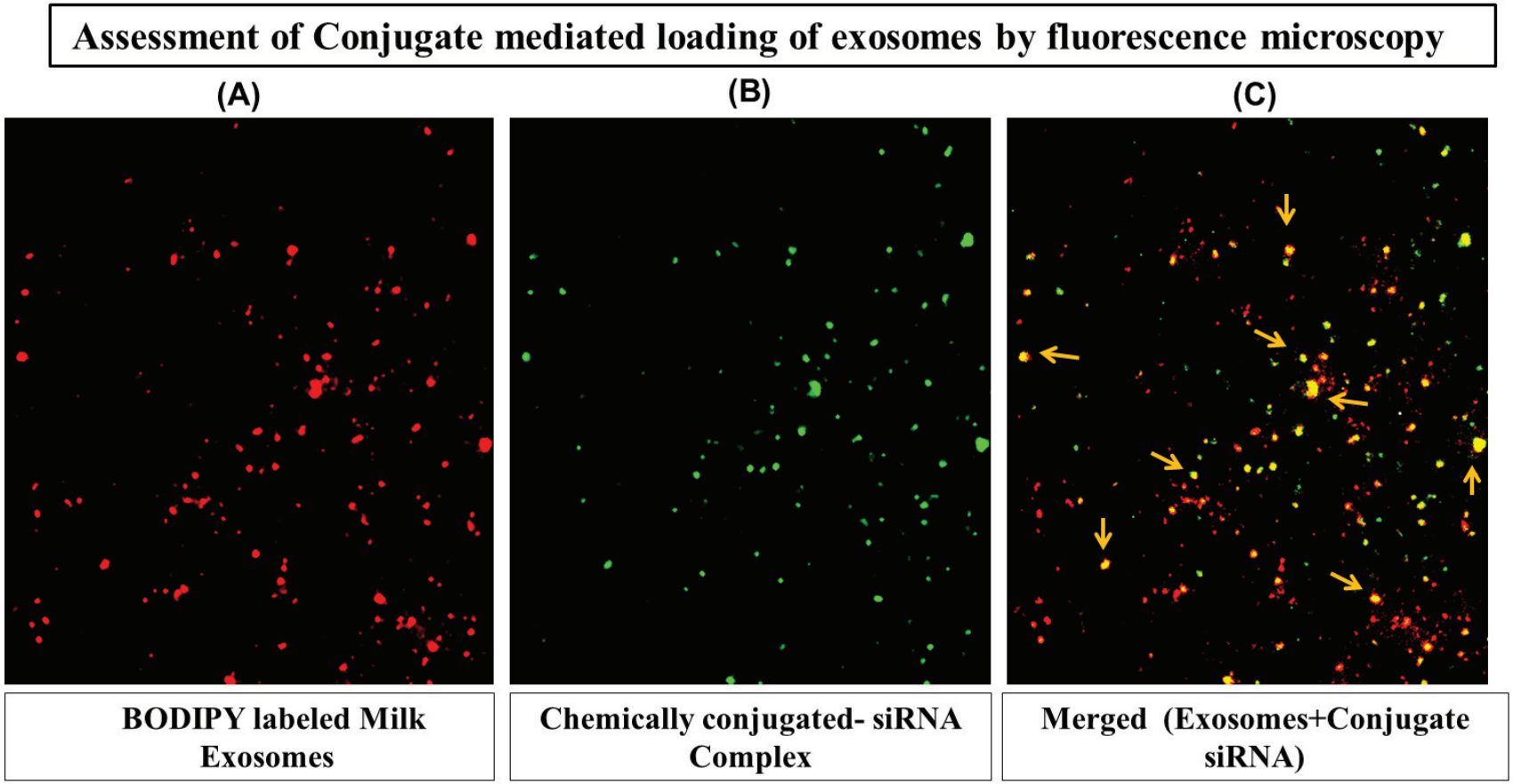


Fig. 4.6 Representative fluorescence microscopy images of milk exosomes loaded with AF-488siRNA via chemical conjugate (A) BODIPY®TR ceramide labeled milk exosomes (red fluorescence), (B) chemically conjugated-siRNA (green fluorescence) and (C) Merged image of milk exosome and AF-488 siRNA (red + green = Yellow) showing the encapsulation of chemically conjugated-siRNA in exosomes (n=3).

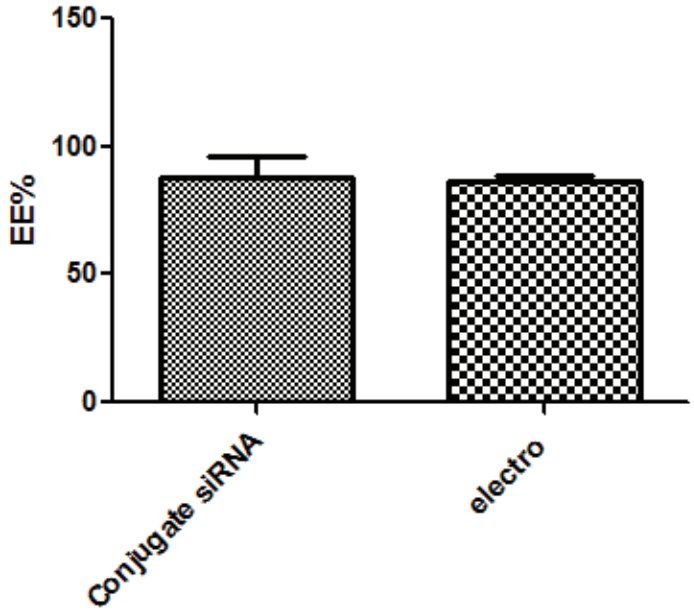


Fig. 4.7 Comparison of encapsulation efficiencies between chemical conjugate mediated loading and electroporation. Y-axis represents the encapsulation efficiency and X-axis represents the conjugate siRNA and electroporated exosomes, respectively (P>0.05, n=3).

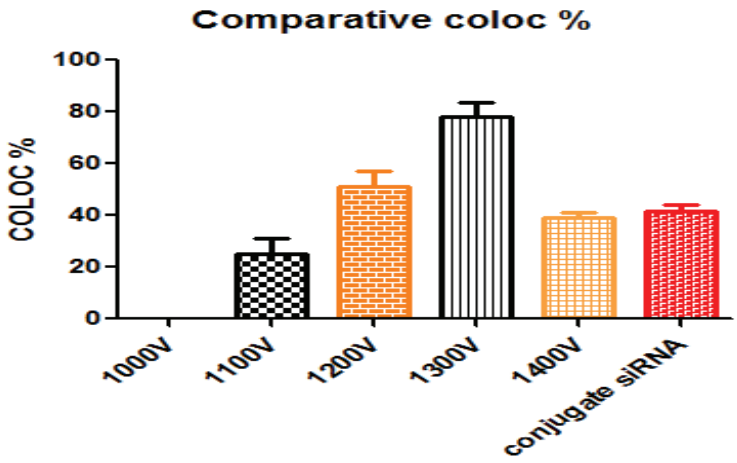


Fig. 4.8 Comparison of colocalization percentage between chemical conjugate mediated loading and electroporation. Y-axis represents the colocalization percentage and X-axis represents electropration at different voltages with range of 1000 V- 1400 V alongwith conjugate siRNA, repectively .P<0.05 for electroporation at 1200 V and chemical conjugate mediated loading of siRNA in exosomes (n=3).

	Rtotal	Rcoloc	R<Thr	M1	M2	Summary
Electroporation at different voltages: 1000V	0.000	0.000	0.000	0.000	0.000	No colocalization
1100V	0.518	0.328	-0.002	0.624	0.767	A moderate uphill positive linear relation
1200V	0.55	0.402	-0.003	0.658	0.845	A moderate uphill positive linear relation
1300V	0.774	0.482	0.001	0.731	0.829	A strong uphill positive linear relation
1400V	0.315	0.282	0.000	0.17	0.90	A weak uphill positive relation
Conjugate siRNA	0.791	0.482	0.001	0.630	0.985	A strong uphill positive linear relation

Table 4. Comparison of colocalization thresholds between electroporation at different voltages and conjugate mediated loading of milk exosomes. Rtotal, Rcoloc and R< threshold represents Pearson's correlation coefficient. M1 and M2 represents Mander's colocalization coefficient. A strong uphill positive correlation was found for both electroporation at 1300 V and for chemical conjugate mediated loading of siRNA in exosomes ($P<0.05$, $n=3$).

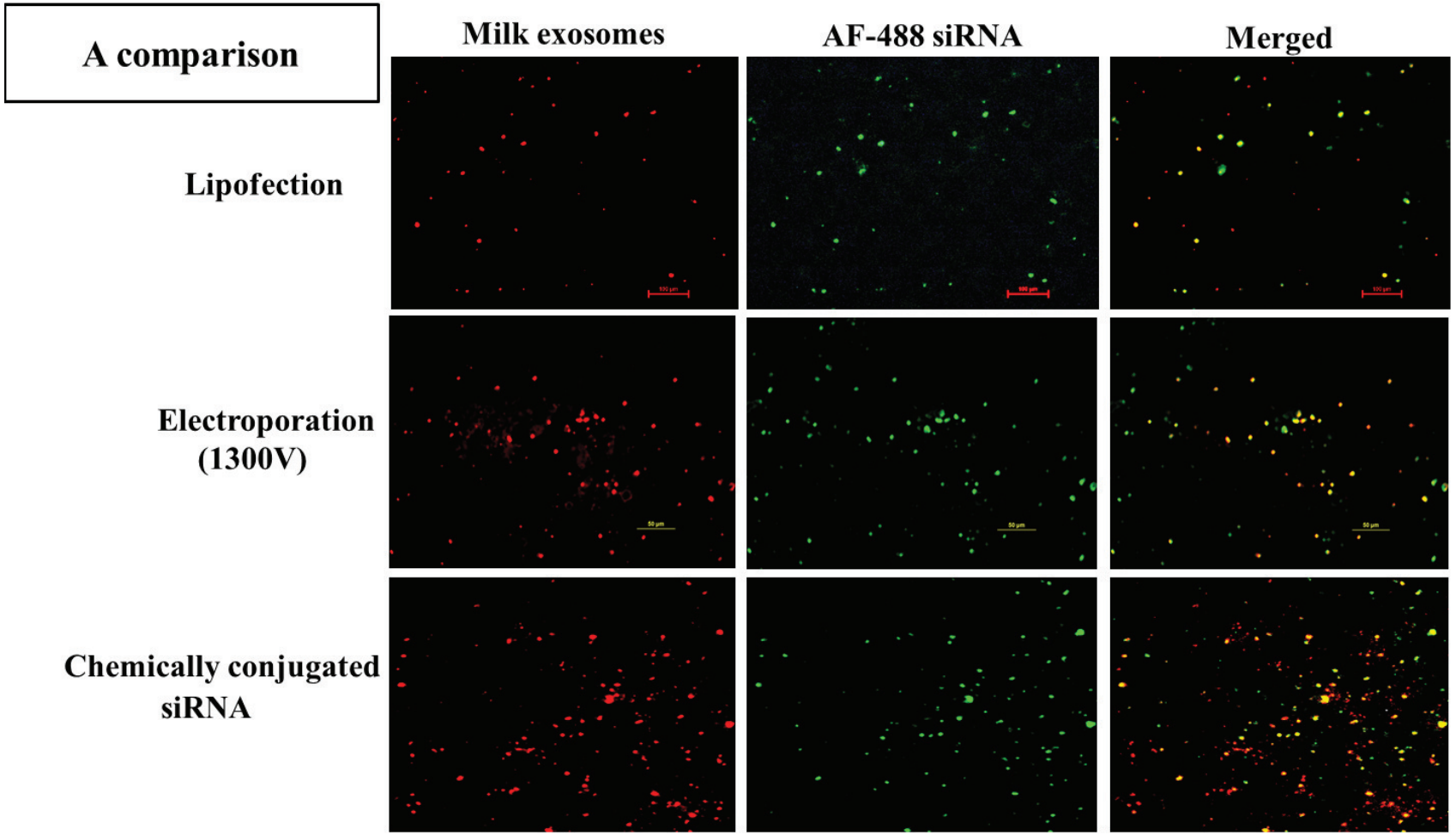


Fig. 4.9 Comparison of fluorescence microscopy images of milk exosome encapsulating AF-488 siRNA. Red- BODIPY®TR Ceramide Labeled milk exosomes, Green- AF-488 siRNA, Yellow- milk exosome encapsulating AF-488 siRNA after lipofection, electroporation (1300 V) and chemical conjugate mediated loading, respectively (n=3).

exosomes encapsulated with siRNA by lipofection, electroporation and conjugate mediated loading has been shown in Fig. 4.9. Comparative analyses suggests that novel developed chemical conjugation mediated loading was as efficient as that of electroporation. Similarly, sonication has been recently reported as an alternative approach of loading HER2 siRNA in exosomes in comparison to electroporation (Lamichhane *et al.,* 2016). To the best of our knowledge, no other alternative approaches of loading siRNA in exosomes other than sonication have been reported and both required costly equipements. Thus, chemical conjugate mediated loading of siRNA in exosomes may serve as an alternative, cost effective and natural method of loading.

4.1.5 *In vitro* digestion of milk exosomes encapsulating AF-488 siRNA

To test if siRNA encapsulated exosome can be delivered orally, milk exosomes encapsulating AF-488 siRNA was subjected to the *in vitro* digestion. The present results showed that the significant proportion of exosomes encapsulating siRNA survived *in vitro* digestion (Fig. 4.10). Although, there was a subsequent loss of milk siRNA encapsulated exosomes when compared to its water control. A comparison of measured fluorescence intensity between *in vitro* digested exosomes and undigested exosomes (water control) has been shown in Fig. 4.10. Fluorescent measurement intensities at excitation wavelength of 490nm and emission wavelength of 525 nm showed a loss of *in vitro* digested exosomes encapsulating siRNA in comparison to water control. In accordance to the results obtained, similar observations were obtained previously (Lonnerdal *et al.,* 2015) They demonstrated that the human milk exosomes were found to be capable of surviving digestion and affects the gene expression after being taken by the enterocytes . Similarly, microRNAs encapsulated in milk exosomes were found to resist *in vitro* digestion and could cross intestinal barrier efficiently (Rani *et al.,* 2017). It was also observed that exosome encapsulation increases the ability of curcumin to sustain *in vitro* digestion process and to cross the intestinal barrier when compared to free curcumin (Vashisht *et al.,* 2017).

4.2 Analysis of siRNA delivery using appropriate cell or cell lines

To test if siRNA encapsulated siRNA can be up taken by cells, cellular uptake of milk exosomes encapsulating siRNA in Caco2 and HepG2 cells were

studied. As demonstrated earlier in our lab, maximum uptake of exosomes in Caco-2 cells occurs after 2 hours of incubation (Rani *et al.,* 2017). The successful cellular uptake and localization of milk exosomes encapsulating AF-488 siRNA in both Caco2 and HepG2 cells was performed. Fluorescence microscopy was done after two hours of exosome-siRNA uptake in Caco2 cellswhereas in HepG2 cells exosome, uptake occurred after 24 hours of treatment. Fluorescence and confocal microscopy images showing the delivery of exosome encapsulating siRNA *in vitro* is shown in Fig. 4.11 and Fig. 4.12, Fig. 4.13, Fig. 4.14, and Fig. 4.15, respectively. These observations confirmed the stable, nontoxic transfection strategy of AF-488 siRNA through milk derived exosomes *in vitro*. Our results are well corroborate with previous studies where similar uptake and localization of exosome loaded with siRNA by lipofectamine has been observed. For instances, a considerable reduction of *RAD51* and *RAD52* protein levels was observed in HeLa and HT1080 cells after transfection with specific siRNA chemically loaded in exosomes (Shatm *et al.,* 2013). High tranfection efficiency of targeted MSP exosomes and RVG exosomes enacapsulating GAPDH and *BACE1* siRNA were observed in murine muscle (C2C12) and Neuronal cells (Neuro2A), respectively. High delivery efficiency was confirmed by fluorescence microscopy (Alvarez-Erviti *et al.,* 2011). Strong fluorescence of cy5 siRNA encapsulated in exosome mimic was observed in tumor xenograft mouse model, BALB/C-nu mice by *in vivo* imaging as compared to free cy5 siRNA (Yang *et al.,* 2016). Endothelial cell exosome loaded with luciferase siRNA by lipofection were found to suppress the luciferase expression in primary endothelial cells (Banizs *et al.,* 2014). siRNA delivered through RVG exosomes were found to be taken up by Neuro2A cells, which reduced the expression level of *MOR* in order to treat the Morphine relapse (Liu *et al.,* 2015). On the similar grounds, fluorescence microscopy confirmed the efficient delivery of siRNA by targeted exosomes in mouse brain *in vitro* and *in vivo* (El-Andaloussi *et al.,* 2012). Comparing with these studies milk exosomes loaded with chemically conjugated siRNA, developed in the present study, was found to be efficiently and stably taken by the Caco2 and HepG2 cells.

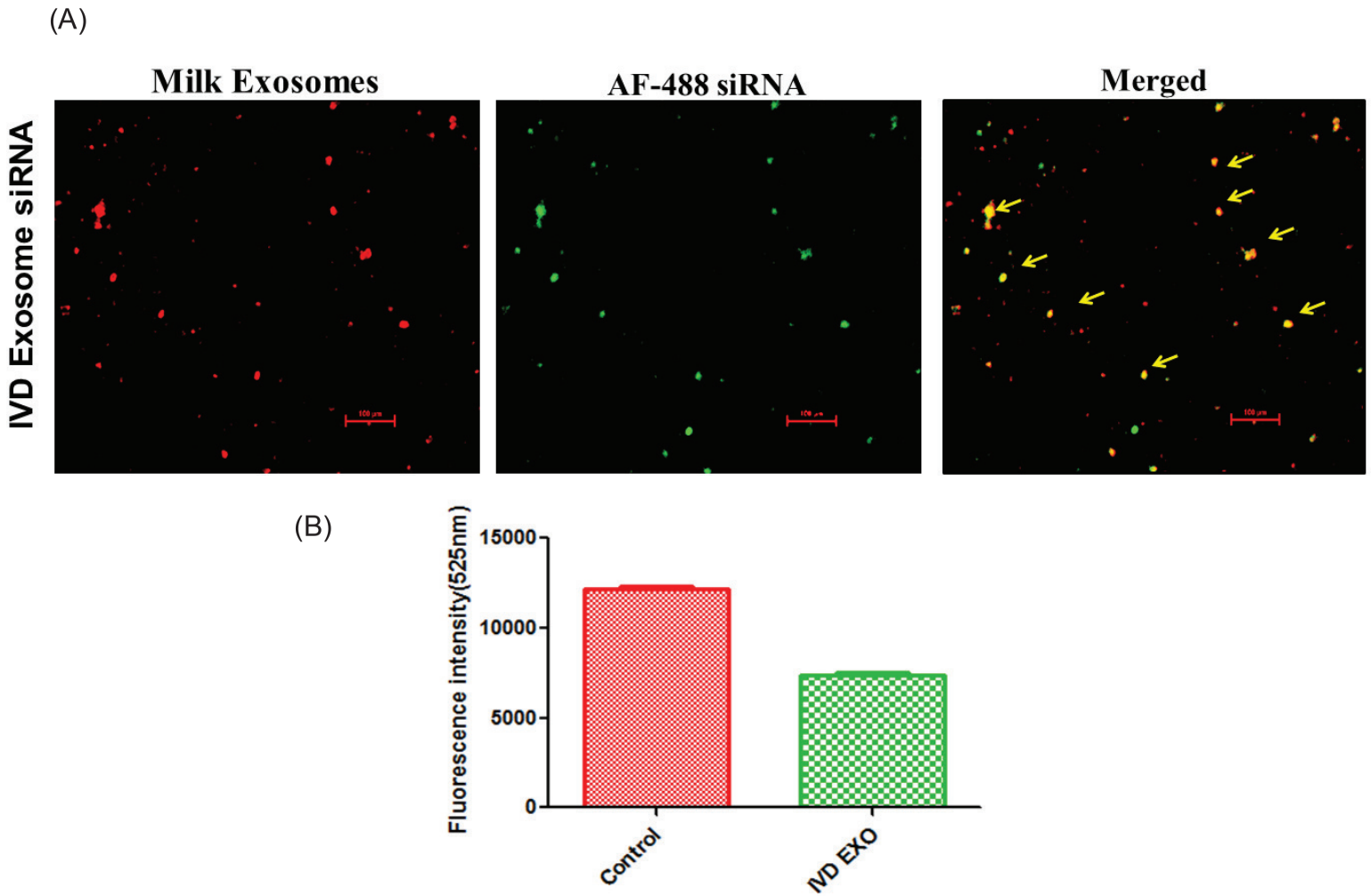


Fig. 4.10 *In vitro* digestion of milk exosomes encapsulating AF-488 siRNA (A) Representative fluorescence microscopy images of exosomes encapsulating siRNA after *in vitro* digestion. Red- BODIPY®TR ceramide labeled milk exosomes, Green-AF-488 siRNA, Yellow- Merged image of exosome + siRNA, (B) Comparison of fluorescence intensity of *in vitro* digested exosome-siRNA at excitation wavelength of 490 nm and emission wavelength of 525 nm in comparison to water control ($P<0.0001$, $n=3$).

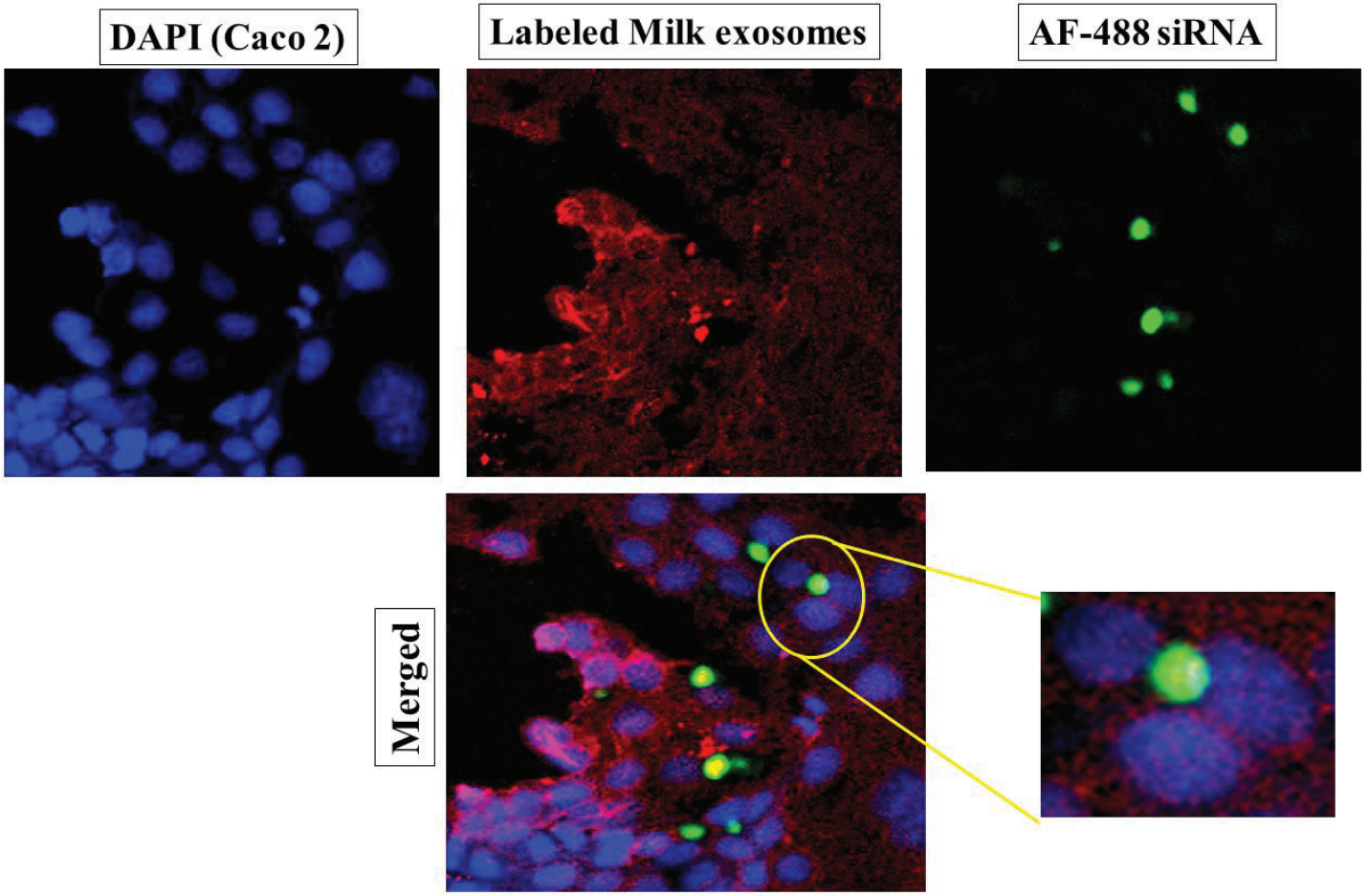


Fig. 4.11 Uptake of milk exosomes encapsulating AF-488 siRNA in Caco2 cells. Fluorescence microscopy images showing the uptake and intercellular localization of electroporated milk exosomes encapsulating siRNA. Nuclei were stained with DAPI (Blue), exosomes were stained with BODIPY®TR Ceramide (Red) and Green represents AF-488 siRNA (Scale bar-40 µm). Merged image represents the uptake of milk exosomes encapsulating siRNA in the Caco2 cells.

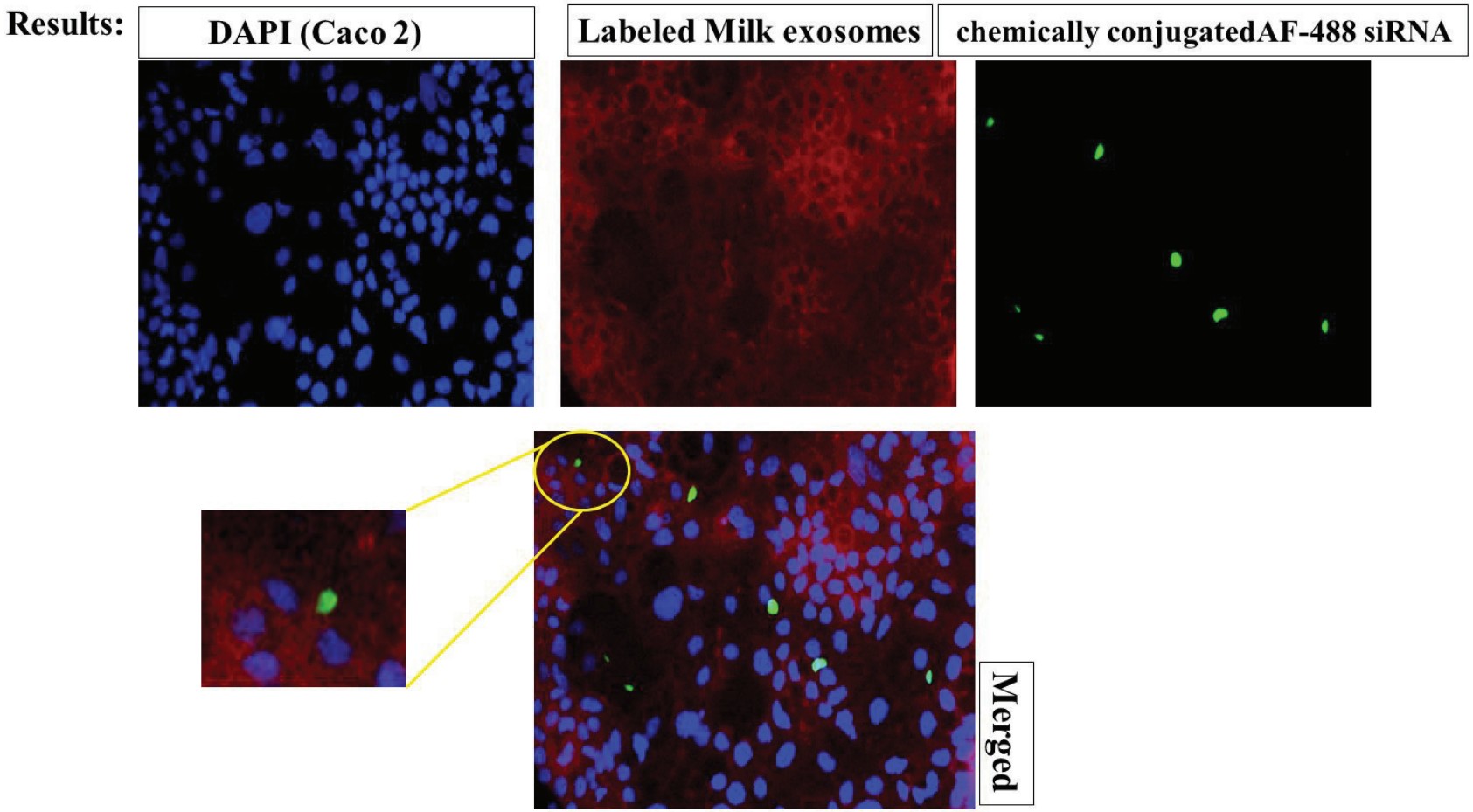


Fig. 4.12 Uptake of milk exosomes encapsulating AF-488 siRNA by chemical conjugation in Caco2 cells. Fluorescence microscopy images showing the uptake and intercellular localization of milk exosomes encapsulating siRNA by chemical conjugation. Nuclei were stained with DAPI (Blue), exosomes were stained with BODIPY®TR Ceramide (Red) and Green represents AF-488 siRNA (Scale bar-40 µm). Merged image represents the uptake of milk exosomes encapsulating siRNA in the Caco2 cells.

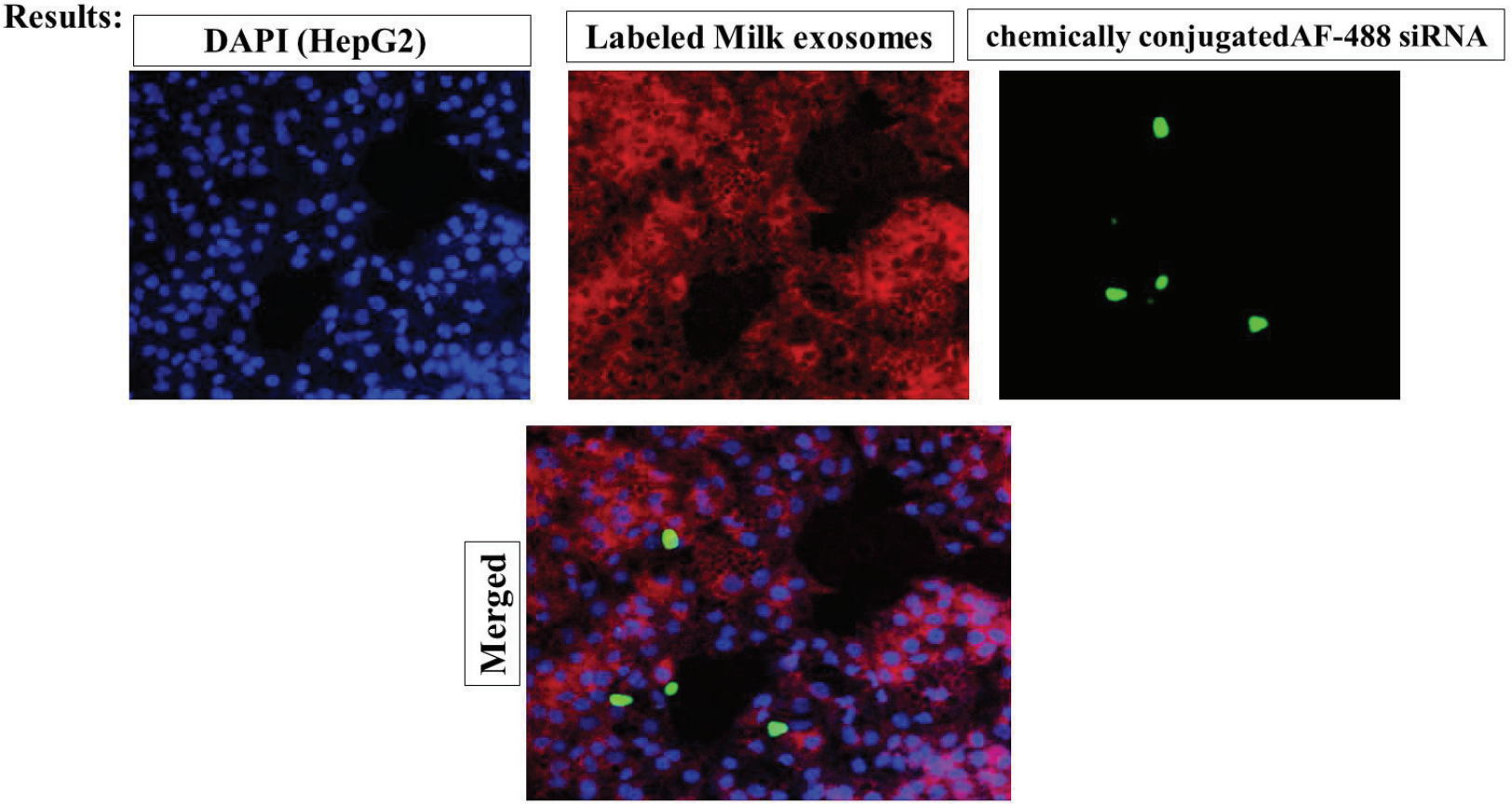


Fig. 4.13 Uptake of milk exosomes encapsulating AF-488 siRNA in HepG2 cells. Fluorescence microscopy images showing the uptake and intercellular localization of milk exosomes encapsulating siRNA by chemical conjugation. Nuclei were stained with DAPI (Blue), exosomes were stained with BODIPY®TR Ceramide (Red) and Green represents AF-488 siRNA (Scale bar-40 μm). Merged image represents the uptake of milk exosomes encapsulating siRNA in the HepG2 cells.

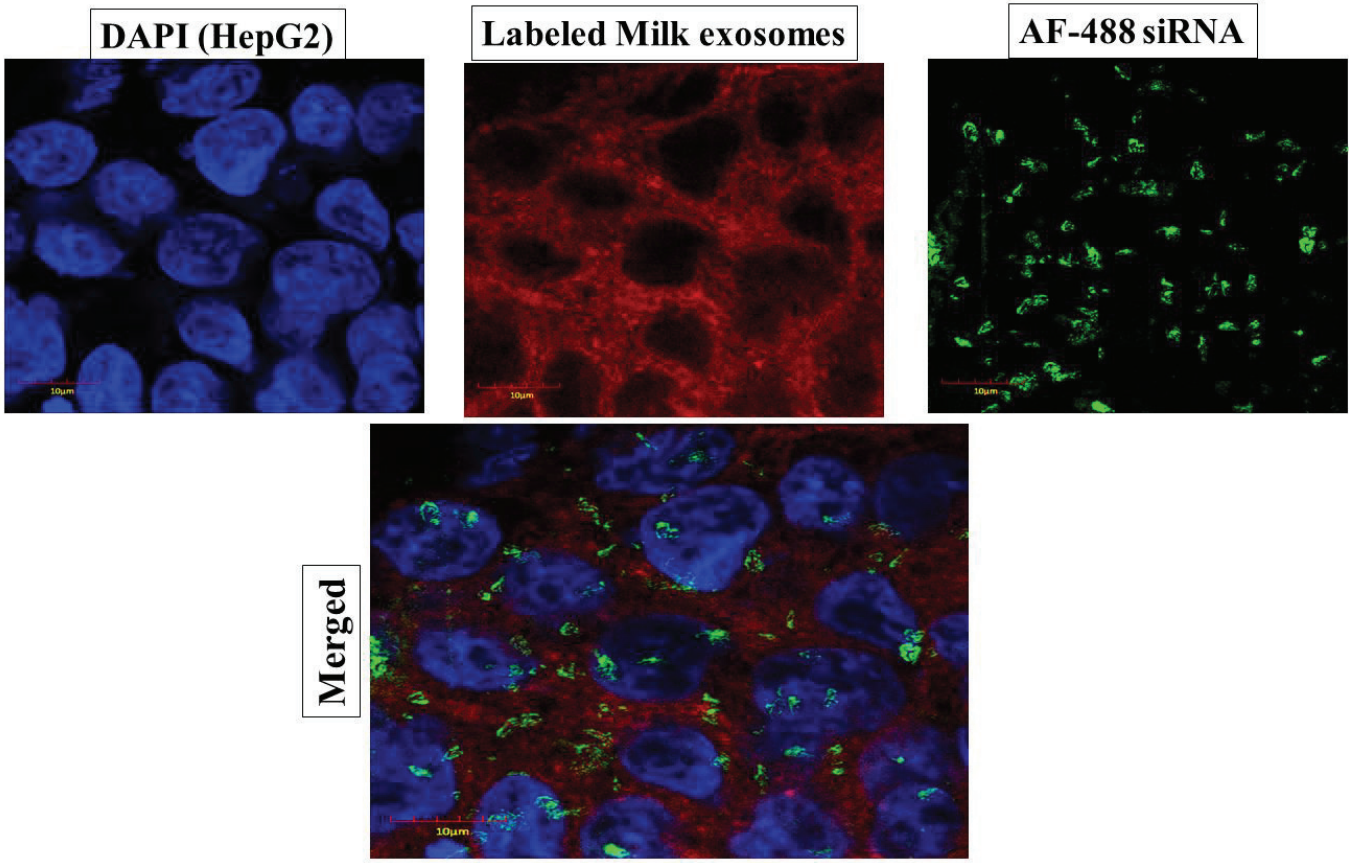


Fig. 4.14 Uptake of milk exosomes encapsulating AF-488 siRNA in HepG2 cells. Confocal microscopy images showing the uptake and intercellular localization of electroporated milk exosomes encapsulating siRNA. Nuclei were stained with DAPI (Blue), exosomes were stained with BODIPY®TR Ceramide (Red) and Green represents AF-488 siRNA (Scale bar-10 µm). Merged image represents the uptake of milk exosomes encapsulating siRNA in the HepG2 cells.

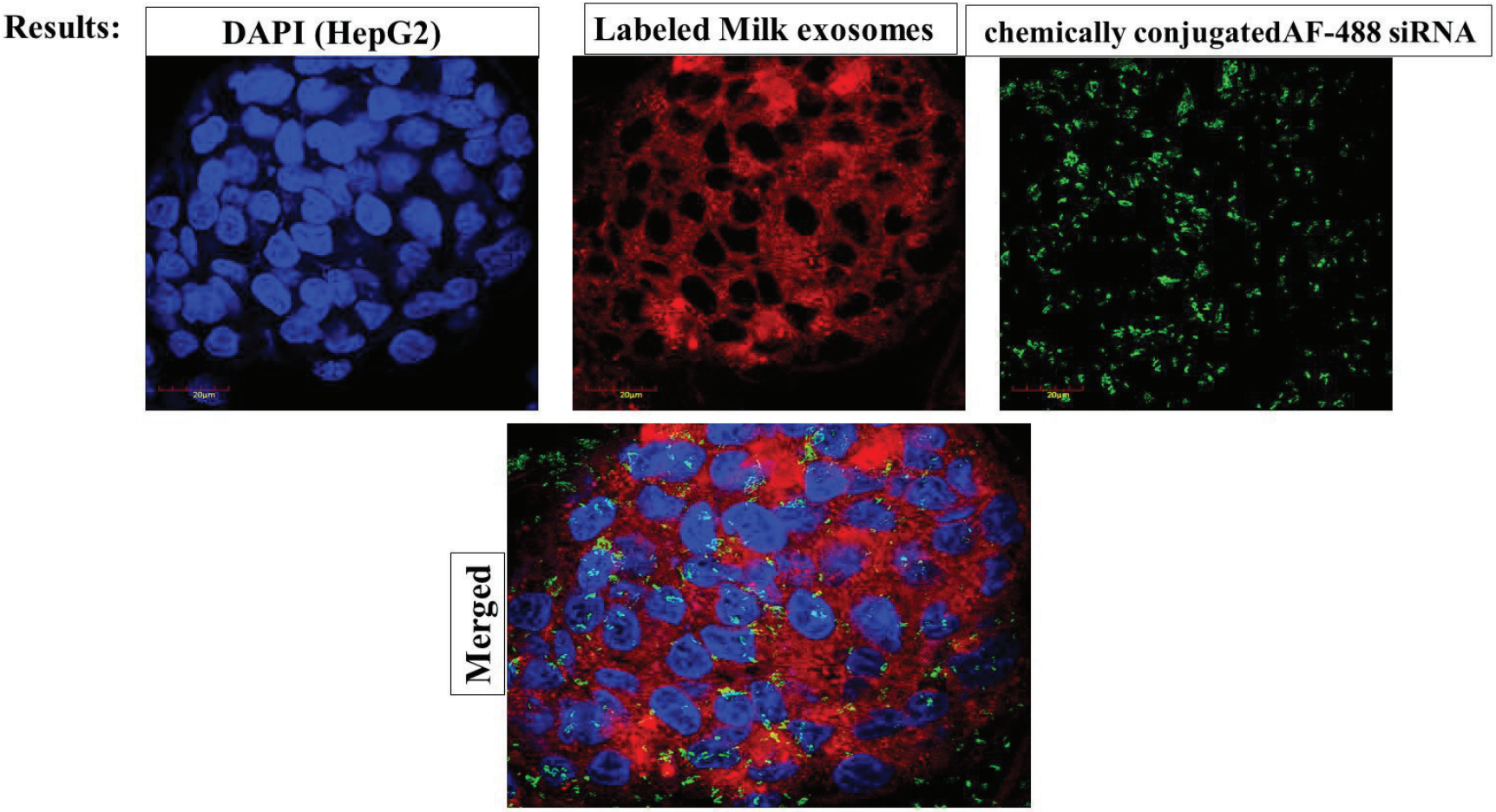


Fig. 4.15 Uptake of milk exosomes encapsulating AF-488 siRNA in HepG2 cells. Confocal microscopy images showing the uptake and intercellular localization of milk exosomes encapsulating siRNA by chemical conjugation. Nuclei were stained with DAPI (Blue), exosomes were stained with BODIPY®TR Ceramide (Red) and Green represents AF-488 siRNA (Scale bar-10 μm). Merged image represents the uptake of milk exosomes encapsulating siRNA in the HepG2 cells.

4.2.1 Cytoxicity assay

Viability of HepG2 cells were tested in the presence of electroporated and chemical conjugate mediated loaded exosomes as shown in Fig. 4.16. No significant cytotoxicity of chemically conjugated siRNA on cells was observed when compared to the electroporated exosomes as control. Similarly, electroporated exosomes when compared to PBS as a control was found to be nontoxic to the cells. Viability of all the cells were maintained at 90% across all the tested groups with a P value of 0.94 and 0.11 for electroporated and chemical conjugate mediated loading of exosomes (P> 0.05), respectively. These results also showed that milk exosomes cause no potential toxicity to the cells *in vitro*. Toxicity of the milk exosomes in female Sprague-Dwaley rats were studied by Munagla *et al.,* 2016. No changes in biochemical and physiological parameters in rats were observed even after the administration of different doses of exosomes for 15 days (Munagla *et al.,* 2016). This observation confirmed that milk exosomes caused no potential toxicity *in vitro* and *in vivo*. Similarly, it was found that exosomes loaded with siRNA by electroporation causes no significant toxicity in MCF-7 cells. Viability of MCF-7 cells was maintained over 90 % in the presence of different doses of electroporated exosomes (Yang *et al.,* 2016). In contrast to exosomes, transfection by cationic liposomes (Lipofectamine) potentially affected the growth, apoptosis and cell cycle of Capan-2 cells, and required the proper optimization of lipofectamine concentration for gene delivery in cells (Zhong *et al.,* 2008). Though, loading of siRNA by electroporation causes no potential toxicity but this method of loading caused the extensive siRNA aggregation which was assessed by confocal microscopy (Kooijmans *et al.,* 2013). This may lead to wrong interpretation of the amount of siRNA actually loaded in exosomes (Kooijmans *et al.,* 2013). Therefore, these observations suggest that milk exosomes loaded siRNA are nontoxic to the Caco-2 and HepG2 cells as showed by the present study. Moreover observing the limitations of electroporation and lipofection, developed novel chemical conjugate mediated loading of siRNA in exosomes was found to be efficient, nontoxic and biocompatible.

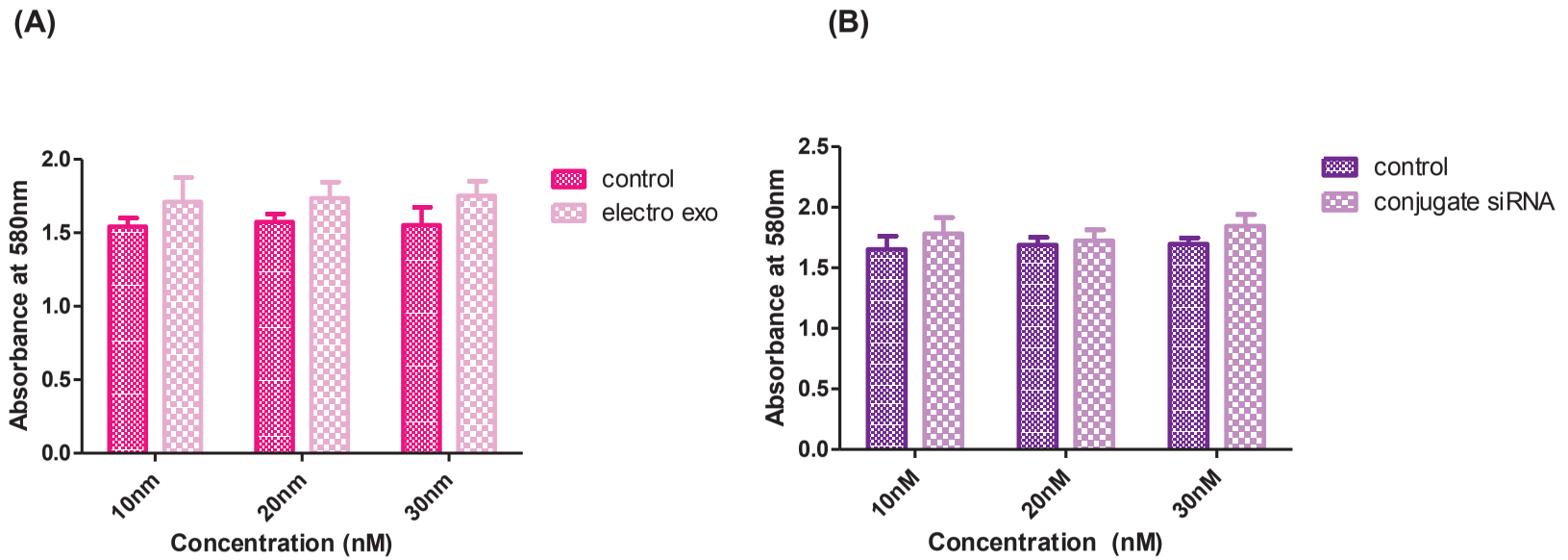


Fig. 4.16 Effect of milk exosome encapsulating AF-488 siRNA on cellular toxicity. Y-axis represents the absorbance at 580 nm and X-axis represents the different concentrations of exosomes encapsulating AF-488 siRNA. (A) Represents the comparison of toxicity of electroporated exosomes encapsulating siRNA to that of PBS as a control ($P>0.05$, n=3). (B) Represents the comparison of toxicity of exosomes encapsulating siRNA by chemical conjugation to that of electroporated exosomes as control ($P>0.05$, n=3).

CHAPTER –5

Summary and Conclusions

SUMMARY AND CONCLUSION

siRNA constitutes a new class of therapeutic modalities known for its high degree of safety and unrestricted choice of targets with a promising clinical efficacy. But the ability of siRNA as therapeutics is greatly hampered by the barriers which come across during its delivery. In order to resolve the delivery problems of siRNA, many delivery systems have been developed till now which includes chemical modifications, lipid and polymer mediated delivery. But these methods of delivery are not full proof particularly unable to minimize the non-specific effects and protection by nuclease digestion. Overcoming these limitations of drug delivery systems used for siRNA delivery, exosomes emerge as a promising drug delivery vehicle. Exosomes are biological nanoparticles secreted in almost all bio-fluids and are known for their property of stable delivery to the target cells due to their natural efficacy of intercellular cargo transport. Thus, utilizing the broad potential of exosomes for safe and efficient delivery of siRNA, use of milk exosome has been demonstrated by several researchers for delivery of siRNA *in vitro*. However, its use for siRNA delivery is limited primarily due to existingloading approaches. In the present study, we compared the methods of loading siRNA in milk exosomes, which included lipofection, electroporation and developed novel chemically conjugated loading method. It was found that chemically conjugate mediated loading of siRNA in milk exosomes had same colocalization percentage, encapsulation efficiency percentage as compared to electroporation based siRNA loading in exosome. It was also observed that colocalization threshold obtained in chemically conjugate mediated loading was comparable to electroporation, showing the strong uphill positive correlation. The previous studies showed that lipofection causes potential toxicity to the cells; electroporation of exosomes causes no potential toxicity but induce aggregation and degradation of siRNA while loading siRNA in exosomes. The present study demonstrated that developed novel chemical conjugate mediated loading of siRNA in exosomes overcomes these limitations of lipofection and electroporation. Results also showed that chemically conjugated siRNA in milk exosomes causes no potential toxicity in HepG2 cells. Further, it was observed that milk exosomes loaded with siRNA by chemical

conjugation was efficiently taken up by the Caco2 and HepG2 cells as assessed by fluorescence and confocal microscopy, showing stable and safe delivery of siRNA via exosomes.

In conclusion, considering factors like availability, cost and toxicity, bovine milk emerge as a suitable natural source for large scale production of exosomes. Biological stability and tolerability make milk exosomes as an effective vehicle to deliver hydrophilic therapeutic small interfering RNA molecule and chemically conjugated method of loading serve as an alternative approach of loading siRNA in exosomes. Chemically conjugated method serves as a cost effective means of loading siRNA in comparison to electroporation, lipofection and sonication. Thus, chemically conjugated method act as a virus free, chemical free, robust, scalable and natural method of loading siRNA in milk derived exosomes. To the best of our knowledge, this is a novel method developed for the first using a biocompatible and cost effective means of loading siRNA in exosomes to enhance oral bioavailability, improve efficacy and safety of siRNA as therapeutics.

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Annexures

APPENDIX-I

LIST OF CHEMICALS

S.No	Chemical	Company
1.	Absolute Ethanol	Merck KGaA, Germany
2.	Agarose	Bangalore Genei, India
3.	Ampicillin, Sodium salt	Himedia Laboratories Pvt. Ltd., India
4.	α-amylase from bacillus licheniformis liquid	Sigma-Aldrich Co., USA
5.	AllStars Negative Control siRNA	Qiagen,Germany
6.	BCA protein assay kit	Pierce™, Thermo scientific
7.	Bile extract Porcine	Santa Cruz Biotechnology, US
8.	Bodipy®TR Ceramide	Molecular probes™, life technologies
9.	Bromophenol Blue	S.d Fine-Chem. Ltd., India
10.	BSA	Sigma-Aldrich Co., USA
11.	Calcium chloride dihydrate	Sigma-Aldrich Co., USA
12.	Chloroform (M.B. Reagent)	Sigma-Aldrich Co., USA
13.	DEPC	Sigma-Aldrich Co., USA
14.	4',6-Diamidino-2-phenylindole (DAPI)	Sigma-Aldrich Co., USA
15.	DMSO	Amresco, USA
16.	Dulbecco's Modified Eagle's Medium	Sigma-Aldrich Co., USA
17.	Electrolytic buffer E2 (Neon® 100µl kit)	Invitrogen by Life technologies
18.	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide	Sigma-Aldrich Co., USA
19.	Ethidium bromide	Sigma-Aldrich Co., USA
20.	ExoQuick-TC™	System biosciences Amresco, USA
21.	Glycine	Bio-Rad Laboratories, USA
22.	Insulin	Sigma-Aldrich Co., USA
23.	Lysozyme from chicken egg white	Sigma-Aldrich Co., USA
24.	L-glutamine	Sigma-Aldrich Co., USA
25.	MilliQ water	Millipore Corporation, USA
26.	miRCURY™	Exiqon

S.No	Chemical	Company
27.	MTT	Sigma-Aldrich Co., USA
28.	Mucin from porcine stomach Type II	Sigma-Aldrich Co., USA
29.	Mucin from porcine stomach type III	Sigma-Aldrich Co., USA
30.	Non-essential amino acids	Sigma-Aldrich Co., USA
31.	Nuclease free water	Ambion, Applied Biosystems, USA
32.	Pancreatin	Sigma-Aldrich Co., USA
33.	Paraformaldehyde	Sigma-Aldrich Co., USA
34.	Pepsin from porcine gastric mucosa	Sigma-Aldrich Co., USA
35.	Penicillin-Streptomycin solution	Sigma-Aldrich Co., USA
36.	Protease free BSA	Hyclone
37.	Resuspension buffer R(Neon®100µl kit)	Invitrogen by life technologies
38.	Sodium chloride (M.B. Reagent)	Sigma-Aldrich Co., USA
39.	Sodium selenite	Sigma-Aldrich Co., USA
40.	Transferrin	Sigma-Aldrich Co., USA
41.	Triton X-100	Bio-Rad Laboratories, USA
42.	Trypsin from bovine pancreas	Sigma-Aldrich Co., USA
43.	Trypan blue dye	Sigma-Aldrich Co., USA
44.	Water sterile tissue culture grade	Himedia Laboratories Pvt. Ltd., India
45.	X-tremeGENE HP DNA Transfection Reagent	Roche Applied Science, Mannheim, Germany

APPENDIX-II

LIST OF EQUIPMENTS

S.No.	Equipment	Brand name
1.	Centrifuge(Table-top)	Remi, India
2.	Deep Freezer (-20°C)	Vestfrost, Finland
3.	Deep Freeze (-80°C)	Thermo Electron Corp., USA
4.	Distillation plant	Scientronic, India
5.	Double beam spectrophotometer	Specord 200 analytikjena, Germany
6.	Elisa plate reader	Eon BioTek
7.	Electron Weighing balance	Sartorius BP11D, France; Shimadzu Corporation(AW220), Japan
8.	Fluorescence microscope(Nikon eclipse Ti)	Nikon, Japan
9.	Fluorescence plate reader	(Infinite®200 PRO, Tecan, Switzerland)
10.	Forma series II Water jacketed Thermo CO_2 Incubator	Electron Corporation, USA
11.	Gel Documentation	Alpha Innotech, USA; BioRad Laboratories Pvt. Ltd., USA
12.	Heating Block	PeqLab, Germany
13.	pH meter	Orion (Thermo electron corporation), USA
14.	Horizontal gel electrophoresis	Peqlab, Germany; Bangalore Genei, India
15.	apparatus	
16.	Icematic machine	Blue star, India
17.	Incubator	Scientronic, India
18.	Incubator-cum-Shaker	MRC, Israel
19.	Inverted Microscope	Olympus, CK2
20.	Ion Coater	Hitachi IB-3, Japan

S.No	Equipment	Brand name
21.	Laminar Flow	BBL Biological Cabinet, USA
22.	Microcentrifuge	Remi, India; PeqLab, Germany
23.	Nanophotometer	Implen, Germany
24.	Neon® Transfection Device	Thermo Fisher scientific
25.	Oven	Akash Deep, India
26.	Refrigerated high speed	MIKRO 22 R, Hetich Centrifuge, Germany;
27.	Mirocentrifuge	Supra 22K Korea
28.	Roto spin-Test tubes rotator	Tarson, India
29.	Scanning electron microscope	EVO® 18, Carl ZEISS special edition, UK
30.	Spiner	Tarson, India; Peq Lab, Germany
31.	Syringes	Dispovan, India
32.	U.V. Transilluminator	VilberLourmant, France
33.	Zetasizer	Malvern Insruments limited, UK