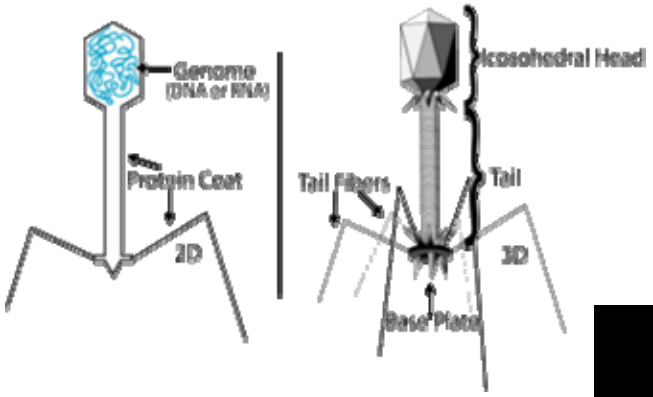
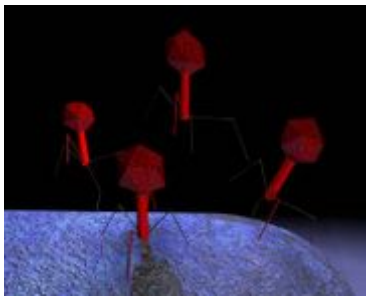
Chapter 21

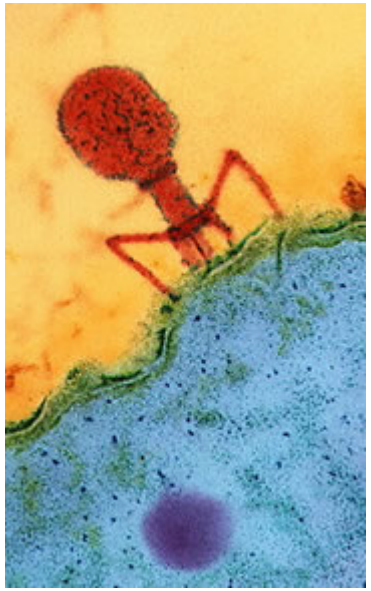
Non-viral Transfection

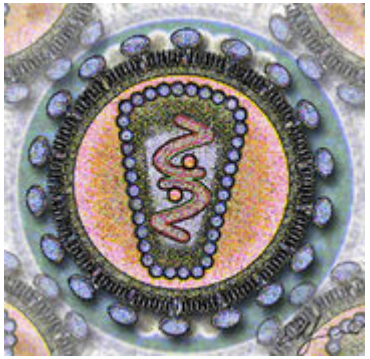
DNA → RNA → Protein

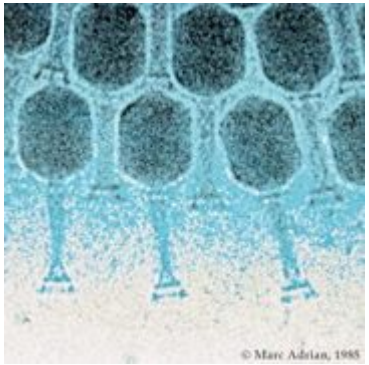
Virus



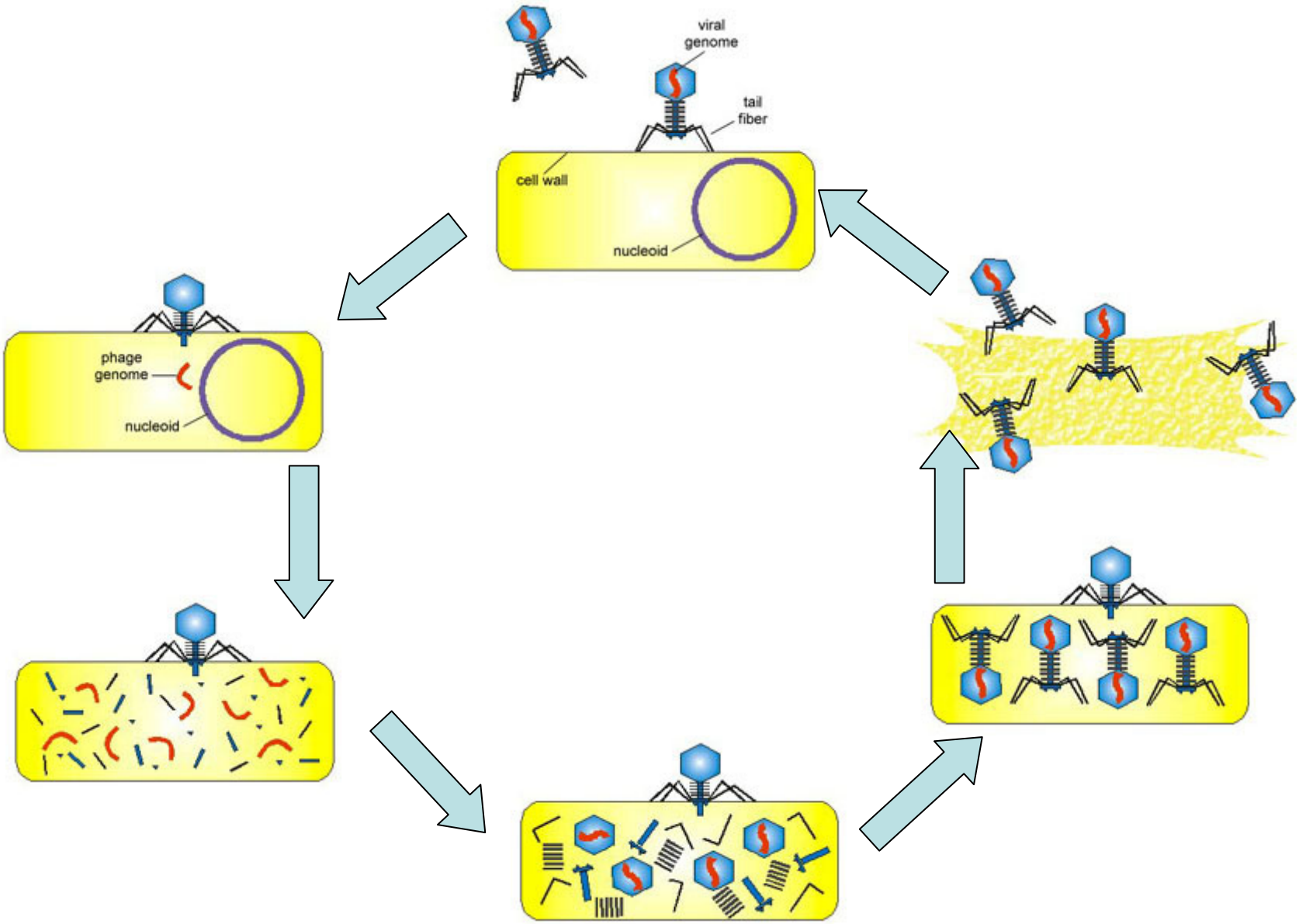








Virus Reproduction

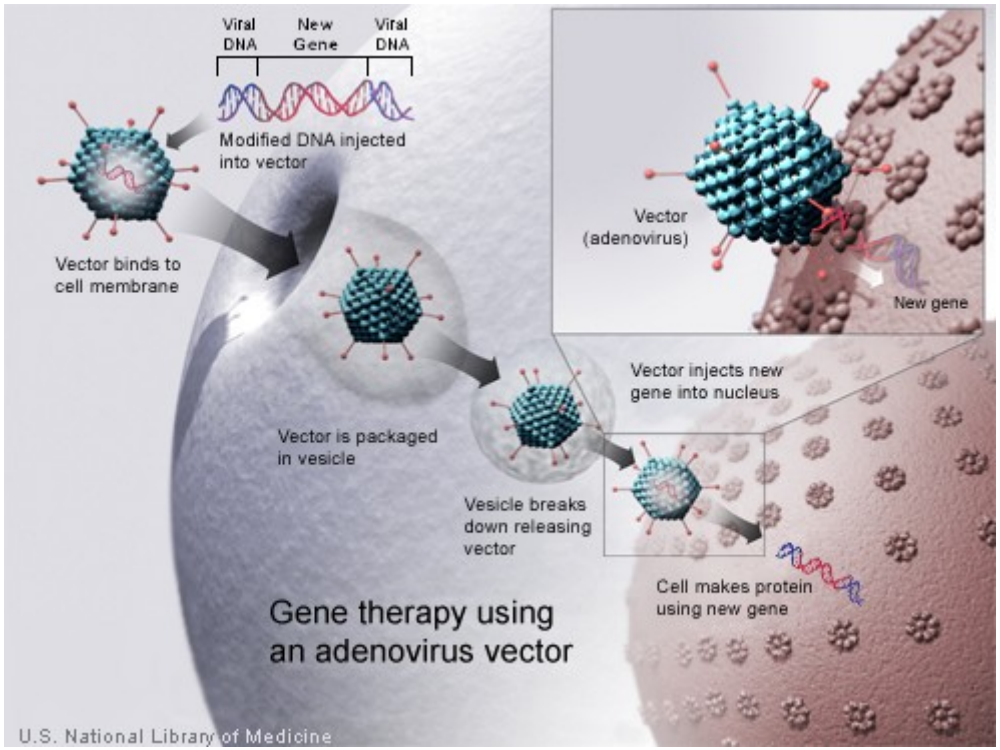


Gene Therapy

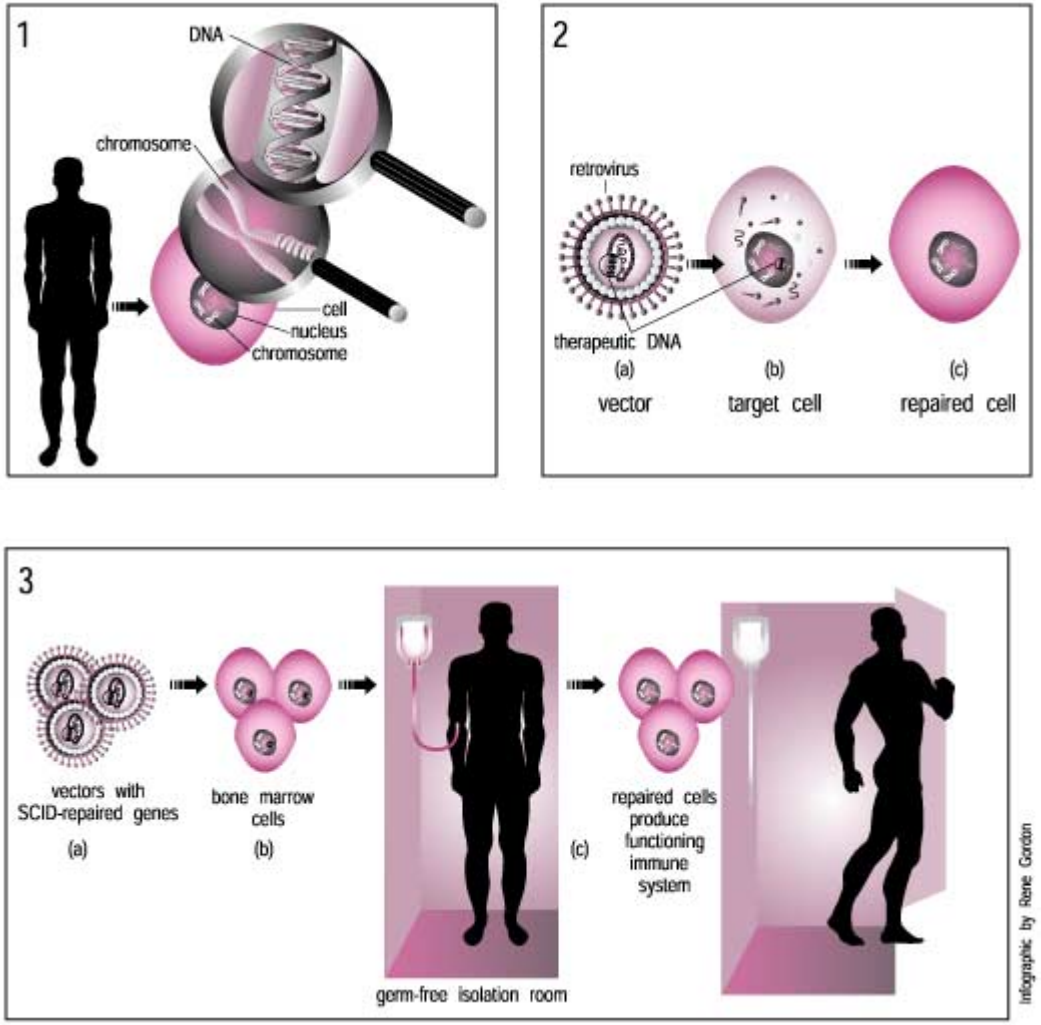
- Gene therapy is a technique for correcting defective genes responsible for disease development. Researchers may use one of several approaches for correcting faulty genes:
  - **A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene. This approach is most common.**
  - **An abnormal gene could be swapped for a normal gene through homologous recombination.**
  - **The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function.**
  - **The regulation (the degree to which a gene is turned on or off) of a particular gene could be altered.**

How Gene Therapy Works?

- In most gene therapy studies, a "normal" gene is inserted into the genome to replace an "abnormal," disease-causing gene. A carrier molecule called a vector must be used to deliver the therapeutic gene to the patient's target cells. Currently, the most common vector is a virus that has been genetically altered to carry normal human DNA. Viruses have evolved a way of encapsulating and delivering their genes to human cells in a pathogenic manner. Scientists have tried to take advantage of this capability and manipulate the virus genome to remove disease-causing genes and insert therapeutic genes.
- Target cells such as the patient's liver or lung cells are infected with the viral vector. The vector then unloads its genetic material containing the therapeutic human gene into the target cell. The generation of a functional protein product from the therapeutic gene restores the target cell to a normal state.


Viral DNA
New Gene
Viral DNA
Modified DNA injected into vector
Vector binds to cell membrane
Vector (adenovirus)
New gene
Vector injects new gene into nucleus
Vector is packaged in vesicle
Vesicle breaks down releasing vector
Cell makes protein using new gene
Gene therapy using an adenovirus vector
U.S. National Library of Medicine

IGF1-1



http://www.fda.gov/fdac/features/2000/gene.html

different types of viruses used as gene therapy vectors

- **Retroviruses** - A class of viruses that can create double-stranded DNA copies of their RNA genomes. These copies of its genome can be integrated into the chromosomes of host cells. Human immunodeficiency virus (HIV) is a retrovirus.

- **Adenoviruses** - A class of viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans. The virus that causes the common cold is an adenovirus.

- **Adeno-associated viruses** - A class of small, single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome 19.

- **Herpes simplex viruses** - A class of double-stranded DNA viruses that infect a particular cell type, neurons. Herpes simplex virus type 1 is a common human pathogen that causes cold sores.

What factors have kept gene therapy from becoming an effective treatment for genetic disease?

- **Short-lived nature of gene therapy** - Before gene therapy can become a permanent cure for any condition, the therapeutic DNA introduced into target cells must remain functional and the cells containing the therapeutic DNA must be long-lived and stable. Problems with integrating therapeutic DNA into the genome and the rapidly dividing nature of many cells prevent gene therapy from achieving any long-term benefits. Patients will have to undergo multiple rounds of gene therapy.

- **Immune response** - Anytime a foreign object is introduced into human tissues, the immune system is designed to attack the invader. The risk of stimulating the immune system in a way that reduces gene therapy effectiveness is always a potential risk. Furthermore, the immune system's enhanced response to invaders it has seen before makes it difficult for gene therapy to be repeated in patients.

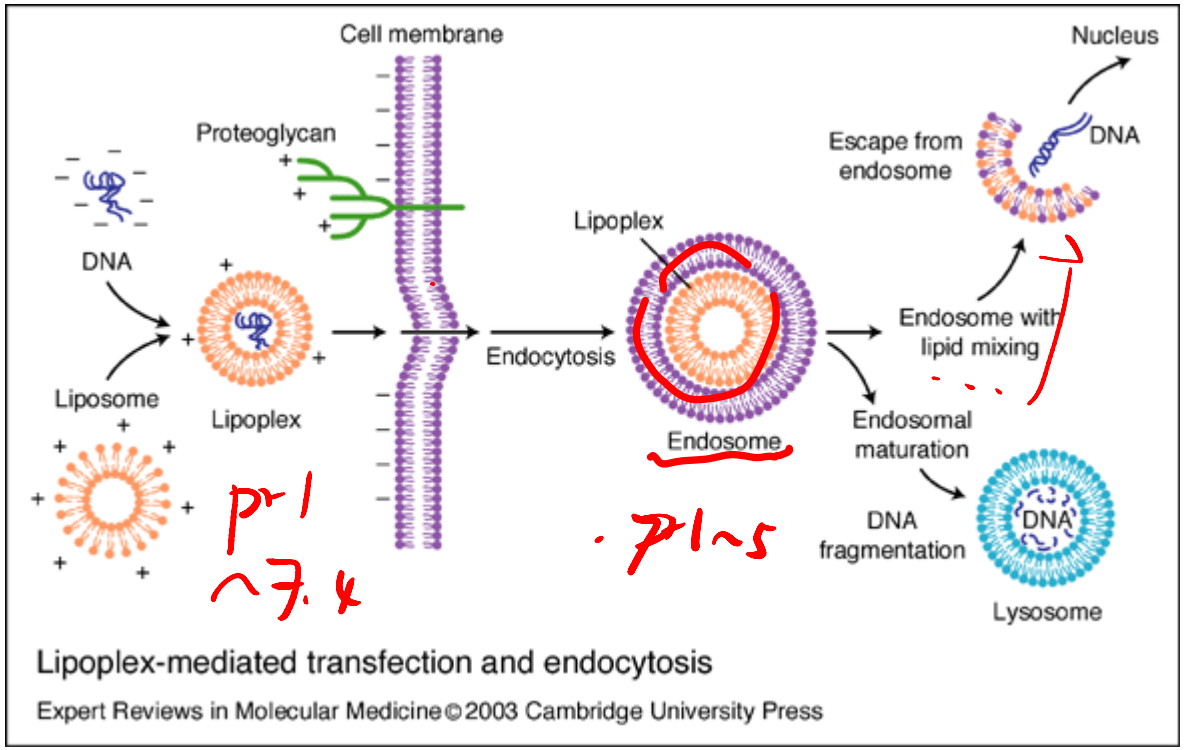
- **Problems with viral vectors** - Viruses, while the carrier of choice in most gene therapy studies, present a variety of potential problems to the patient --toxicity, immune and inflammatory responses, and gene control and targeting issues. In addition, there is always the fear that the viral vector, once inside the patient, may recover its ability to cause disease.

- **Multigene disorders** - Conditions or disorders that arise from mutations in a single gene are the best candidates for gene therapy. Unfortunately, some the most commonly occurring disorders, such as heart disease, high blood pressure, Alzheimer's disease, arthritis, and diabetes, are caused by the combined effects of variations in many genes. Multigene or multifactorial disorders such as these would be especially difficult to treat effectively using gene therapy. For more information on different types of genetic disease

Gene Delivery

- Transfection- the delivery of foreign molecules such as DNA and RNA into eukaryotic cells
- Naked DNA is not suitable for in-vivo transport of genetic materials-> degradation by serum nucleases
- Ideal gene delivery system
  - Biocompatible
  - Non-immunogenic
  - Stable in blood stream
  - Protect DNA during transport
  - Small enough to extravagate
  - Cell and tissue specific

Transfection



1 in 10^6

10^5 cells

$\Rightarrow 10^5 \times 10^6 = $ DNA

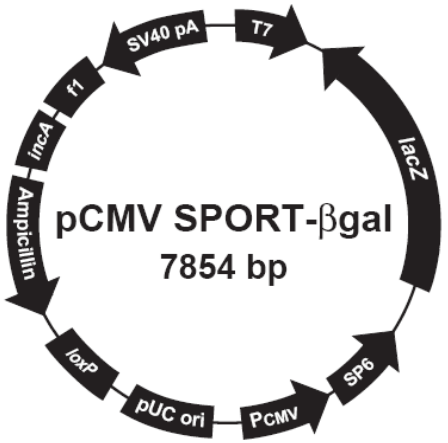
$\Rightarrow$ pmol

$\frac{10^6}{10^{23}} = 10^{-17}$

$= 10$ aM

Transient and Stable Transfection

- Transient
  - No chromosome integration
  - Expression 24-96 Hr
  - Super-coiled plasmid
- Stable
  - Chromosome integration
  - Linear DNA
  - 1 in 10^4
  - Selection



Challenges

- Cell targeting
- Transport through the cell membrane
- Uptake and degradation in endolysome
- Intracellular trafficking of plasmid to nucleus

Transfection Technology

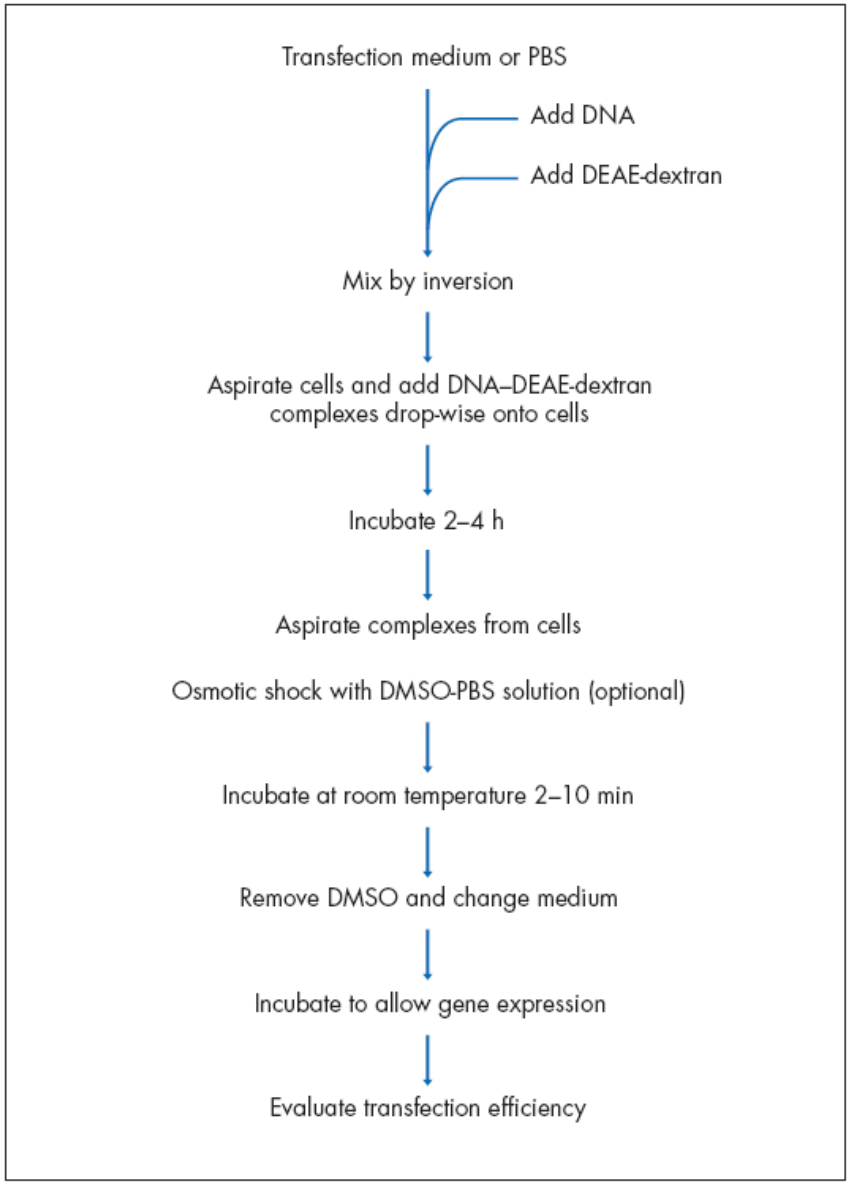
- DEAE dextran
- Calcium phosphate
- Electroporation
- Microinjection
- Biolistic particle
- Nanoparticles
  - Cationic liposome
  - Cationic polyermer
  - Activated dendrimer
  - Gold nanoparticles
  - Chitosan

DEAE-dextran

- Diethylaminoethyl (DEAE)-dextran was introduced in 1965 (5) and is one of the oldest methods for introducing nucleic acids into cultured mammalian cells. The positively charged DEAE-dextran molecule interacts with the negatively charged phosphate backbone of the nucleic acid. The DNA–DEAEdextran complexes appear to adsorb onto the cell surface and be taken up by endocytosis. The advantages of this technique are its relative simplicity and reproducibility of results. Disadvantages include cytotoxic effects and the fact that the amount of serum in the culture medium must be temporarily reduced during the transfection procedure. In addition, the DEAE-dextran method is best suited for transient transfection only.

Dextran is a complex branched polysaccharide made of many glucose molecules joined into chains of varying lengths.

DEAE-Dextran Method*

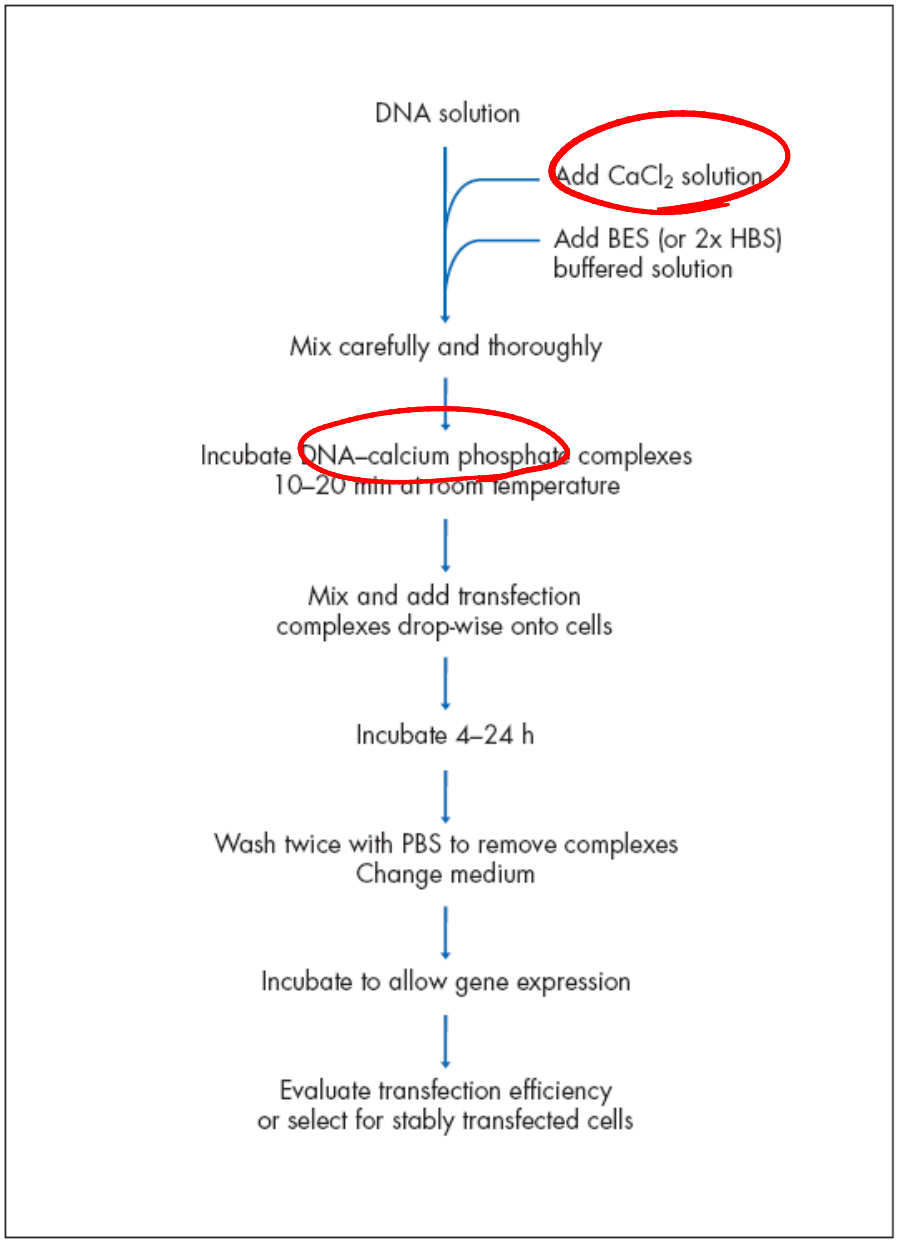


Calcium Phosphate

- The calcium-phosphate method was first used in 1973 to introduce adenovirus DNA into mammalian cells (6). The principle involves mixing DNA in a phosphate buffer with calcium chloride. The resulting calcium-phosphate–DNA complexes adhere to the cell membrane and enter the cytoplasm by endocytosis. Advantages of calcium-phosphate–based transfection are its easy handling and, compared with the DEAE-dextran method, its much higher suitability for stable transfections. However, a common disadvantage is low reproducibility, which is mainly caused by variation in transfection complex size and shape. These variations can be caused by minor changes in the pH of the solutions used for the transfection, as well as the manner in which these solutions are combined. A further drawback of the calcium-phosphate method is that some cell types, including primary cells, may resist this form of DNA transfer.

$CaCl_2$

Calcium-Phosphate Method*



Electroporation

The use of high-voltage pulses to introduce DNA into cultured cells was first established by Wong and Neumann using fibroblasts . Cells in a suitable cuvette are subjected to a short high-voltage pulse that causes the membrane potential of the cells to break down. As a result, pores are formed through which macromolecules such as DNA can enter. The main advantage of electroporation is its applicability for transient and stable transfection of all cell types. However, a disadvantage is that approximately 5-fold greater quantities of DNA and cells are needed than in either DEAE-dextran or calcium phosphate methods. A major drawback of electroporation is the high cell mortality that can result in the death of up to 50–70% of the cells. In addition, the optimal settings for voltage, capacitance, pulse length, and gap width are cell-type dependent, and it is necessary to repeat the electroporation experiment a number of times to optimize the electroporation efficiency and cell viability.

Electroporatior

Pulse voltage:	20-1.200 V
Pulse form:	Exponentially diminishing, electronically controlled
Time constant:	15-500 µs, in increments 5 µs
Multiple pulsing:	1-99, with 1 min. time interval

Cationic Liposome

Liposomes were first introduced in 1987 by Felgner and coworkers (9). The liposomes currently in use typically contain a mixture of cationic and neutral lipids organized into lipid bilayer structures. Transfection-complex formation is based on the interaction of the positively charged liposome with the negatively charged phosphate groups of the nucleic acid. The uptake of the liposome–DNA complexes may be mediated by endocytosis. Compared to the DEAE-dextran and calciumphosphate methods, liposomes often offer higher transfection efficiency and better reproducibility. However, one drawback of liposome-mediated transfection is that the presence of serum during the transfection procedure often lowers the transfection efficiency. For this reason, serum is often omitted when transfecting with liposomes. In many cases, the absence of serum from the medium increases the cytotoxicity of the liposome. Another drawback of classical liposome-mediated transfection is that results

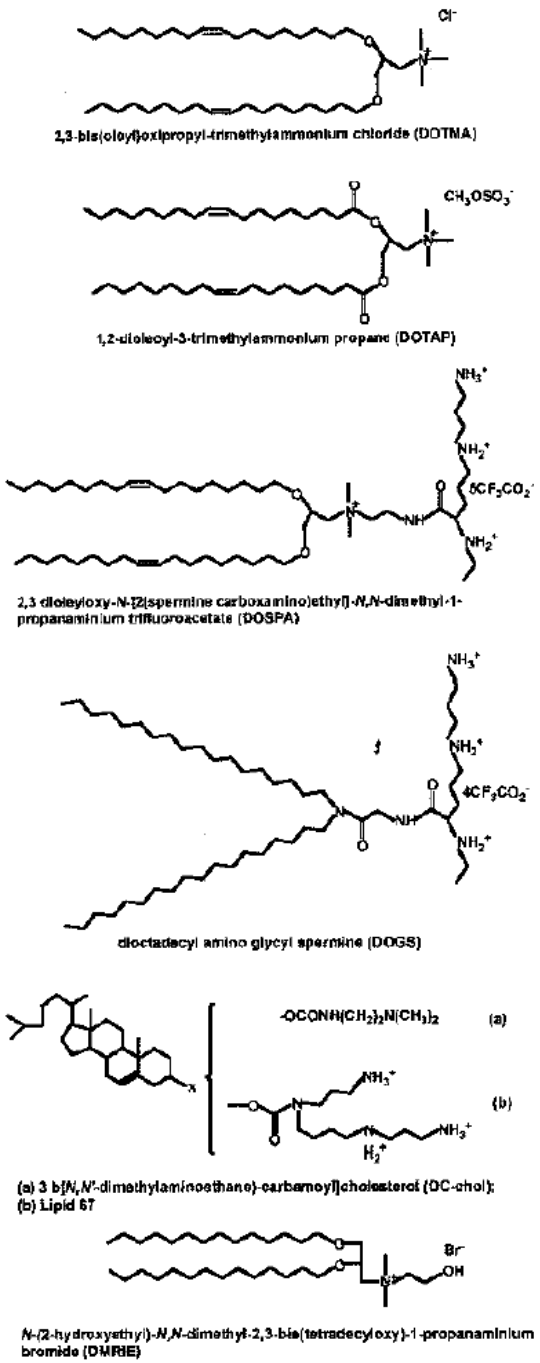
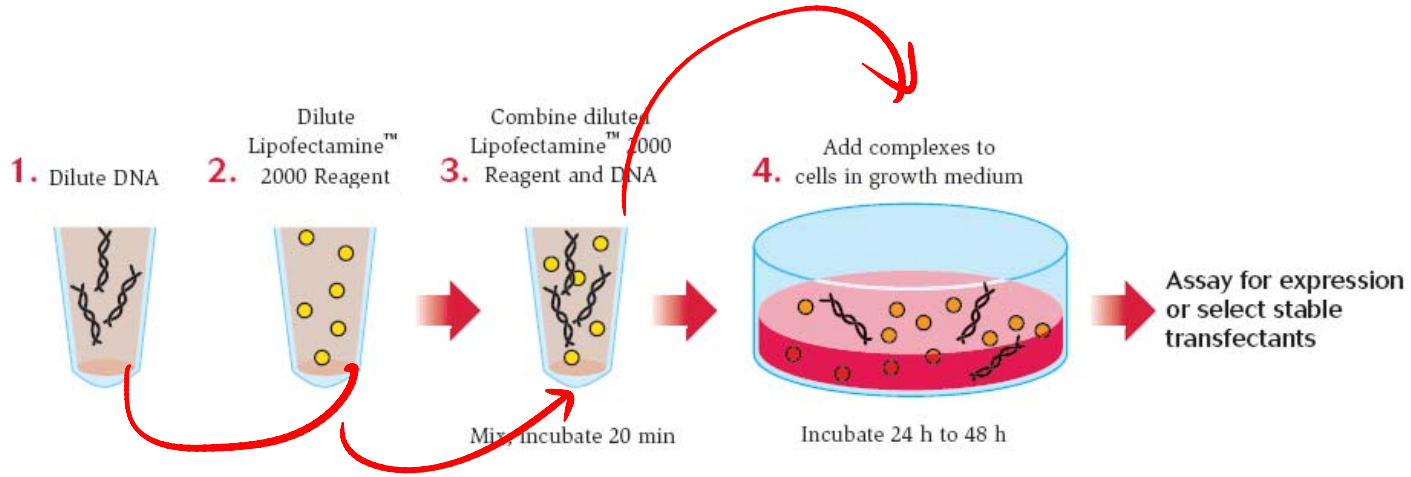
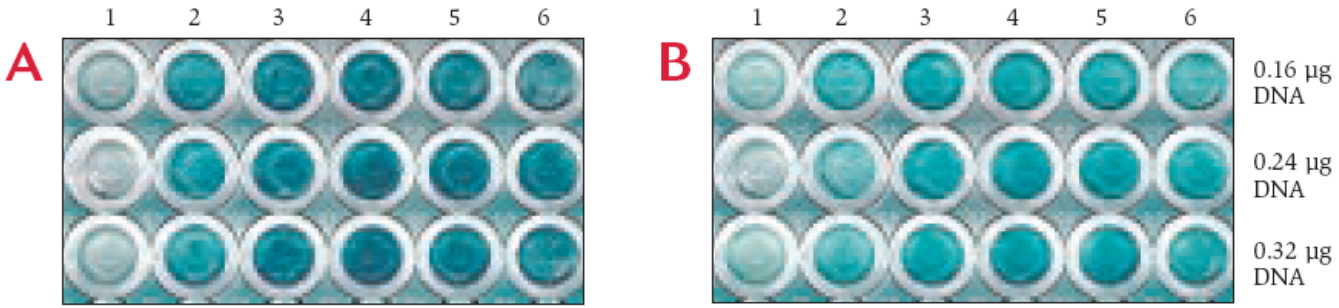


Figure 21.7 Structures of some cationic lipids commonly used in gene therapy.





CHO-S cells were transfected with pCMV•SPORT-βgal DNA (0.16 µg to 0.32 µg) and Lipofectamine™ 2000 Reagent (0.2 µl to 1.2 µl, columns 1-6 respectively) in 96-well plates. After 24 hours, cells were stained with X-gal. **Panel A:** Cells (2 x 10^4) were plated the day before transfection in growth medium containing serum. **Panel B:** The day of transfection, cells were trypsinized, counted, and 5 x 10^4 cells were added directly to the wells containing the complexes.

Cell Line	Cell Type	Transfection efficiency (%)
293-F	Human kidney	99
293-H	Human kidney	99
CHO-S	Hamster ovary	96
COS-7L	Monkey kidney	99
BE(2)C	Human neuroblastoma	77
SKBR3	Human breast cancer	49
MDCK	Dog kidney	43
HT1080	Human fibrosarcoma	81
Human fibroblasts	Primary passaged	48
HeLa	Human cervical carcinoma	94
CV-1	Monkey kidney	70
Vero	Monkey kidney	86
PC12	Rat pheochromocytoma	85
Murine ES	Mouse embryonic stem	75
Rat Hepatocytes	Primary liver	50
E18 Cortical Neurons	Rat primary	25
E18 Hippocampal Neurons	Rat primary	30

β-Galactosidase Enzyme Assay

The Beta-Galactosidase Enzyme Assay System offers a direct and easy procedure for measuring Beta-galactosidase enzyme activity in cells transfected with the pSV-Beta-Galactosidase Control Vector. The pSV-Beta-Galactosidase Vector is designed to be used as a positive control vector for monitoring transfection efficiencies into mammalian cells. Cell extracts are incubated with the provided buffer and substrate ONPG (o-nitrophyl-B-D-galactopyranoside). The optical density is measured spectrophotometrically or with an ELISA reader. The absorbance should be read at 420nm.

$$1 \text{ Miller Unit} = 1000 * (\text{Abs420} - (1.75 * \text{Abs550}))/(t * v * \text{Abs600})$$

where:

Abs420 is the absorbance of the yellow o-nitrophenol,

Abs550 is the scatter from cell debris, which, when multiplied by 1.75 approximates the scatter observed at 420nm,

t = reaction time in minutes,

v = volume of culture assayed in milliliters,

Abs600† reflects cell density.

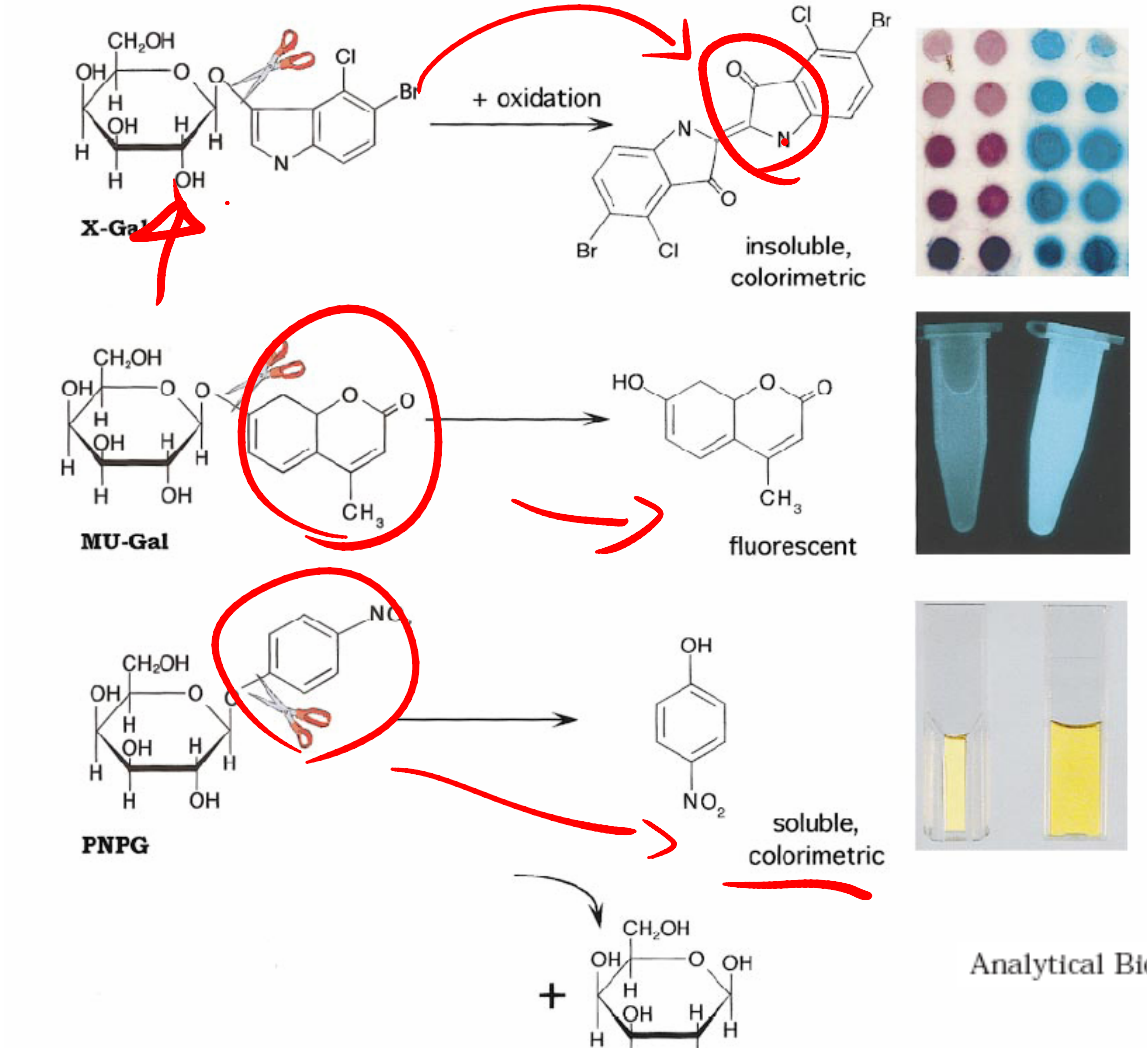
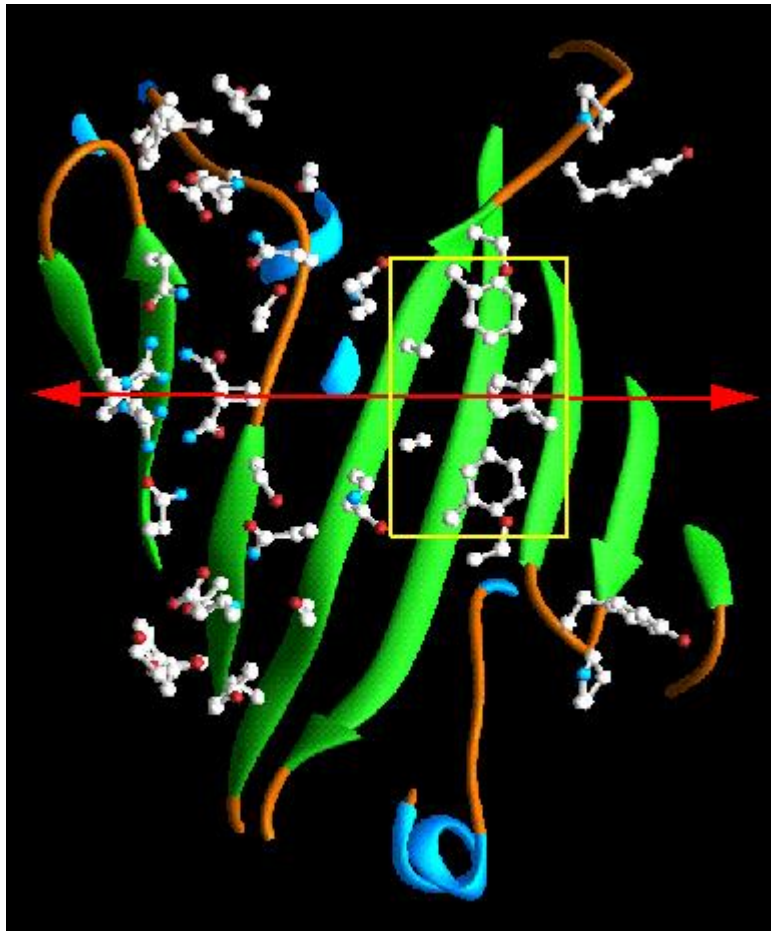
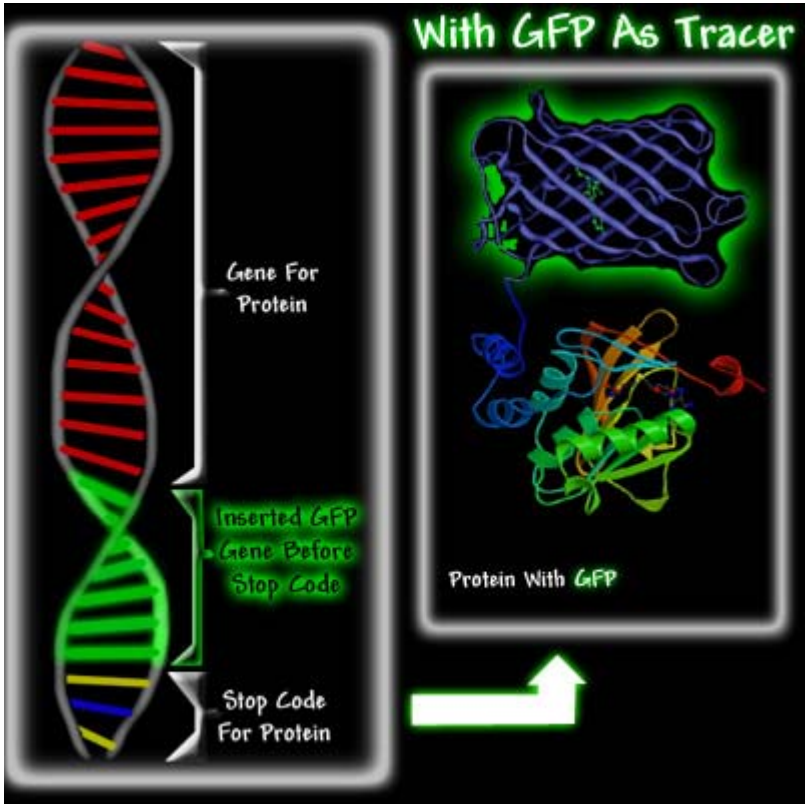


FIG. 1. Enzymatic function of β-galactosidase in cleaving indicator substrates. β-gal cleaves β-D-galactoside containing substrates with a diverse range of aglycone groups, targeting between the glycosyl oxygen and anomeric carbon as indicated (scissors). Substrates shown indicate commonly used indicators for assays on β-gal function on plates (X-Gal) or for liquid assay by measure of fluorescence (MU-Gal or MUG) or color (ONPG). Top left, X-Gal is 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and when cleaved and oxidized produces the insoluble dye 5-bromo-4-chloro-indigo, as described previously (22). Right panel, top, yeast colonies expressing β-gal and exposed to X-Gal (right half) or the closely related compound Magenta-Gal (left half, see Biosynth, Inc., or Diagnostic Chemicals Limited). Middle left, MUG is methylumbelliferyl-β-D-galactoside, and when cleaved by β-gal produces the fluorescent product methylumbelliferone (first described in (102)). Right panel, middle, shows yeast lysates expressing β-gal exposed to MUG, under long-wave UV. Bottom left, PNPG and ONPG are closely related nitrophenol-β-D-galactosides with similar assay properties, e.g., (103), whose cleavage releases the yellow product nitrophenol (right panel, bottom); PNPG is shown.

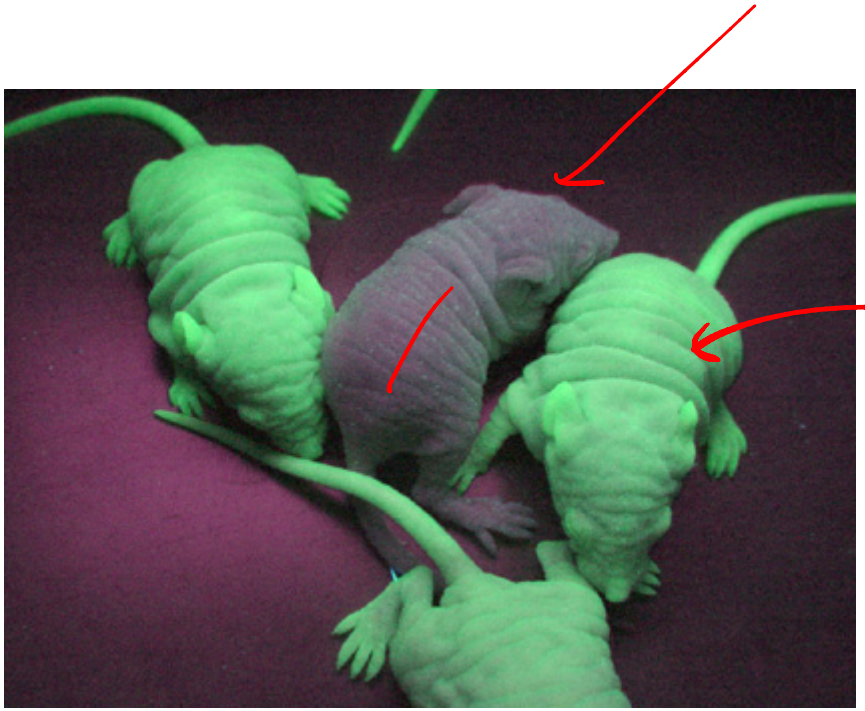
Green Fluorescent Protein (GFP)



The **green fluorescent protein** (**GFP**) is a protein from the jellyfish *Aequorea victoria* that fluoresces green when exposed to blue light.



GFP Rats



Dendrimer

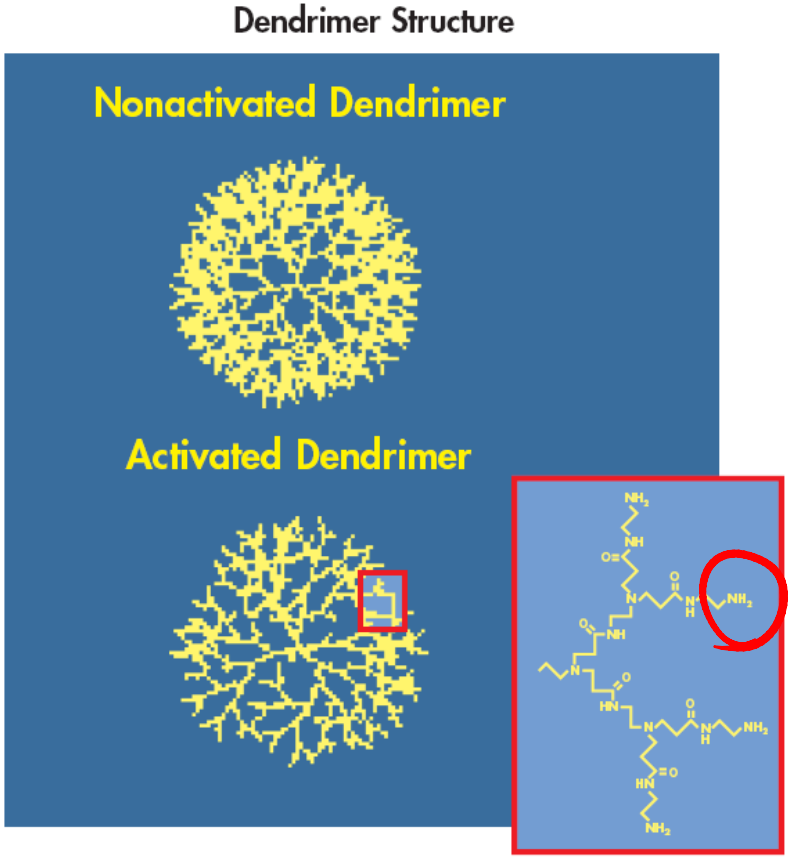


Figure 1. *Schematic diagram of an activated and non-activated dendrimer. A portion of the activated dendrimer molecule is enlarged to show the chemical structure of the molecular branches.*

Activated-Dendrimer–DNA Interaction

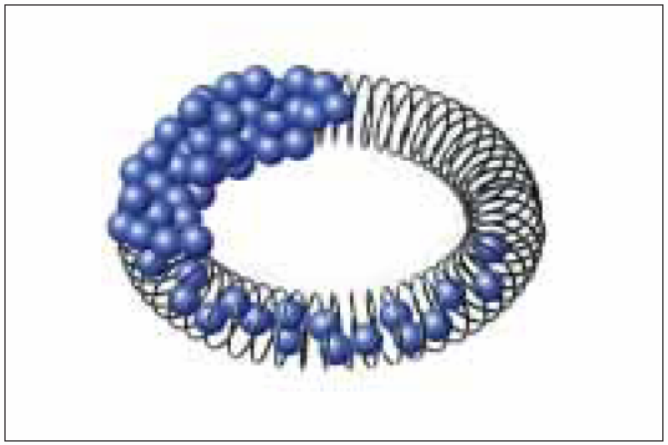


Figure 2. *Model of the activated-dendrimer–DNA complex. Activated dendrimers (purple spheres) interact with DNA (black) to form a ring-like (toroid-like) structure. The upper right section of the illustration shows naked DNA, the lower section shows the interaction between dendrimers and DNA inside the complex, and the upper left section shows the final complete coverage of DNA within the complex.*

β-gal

gfp

green cell

Dem cell

PolyFect Reagent with HeLa Cells

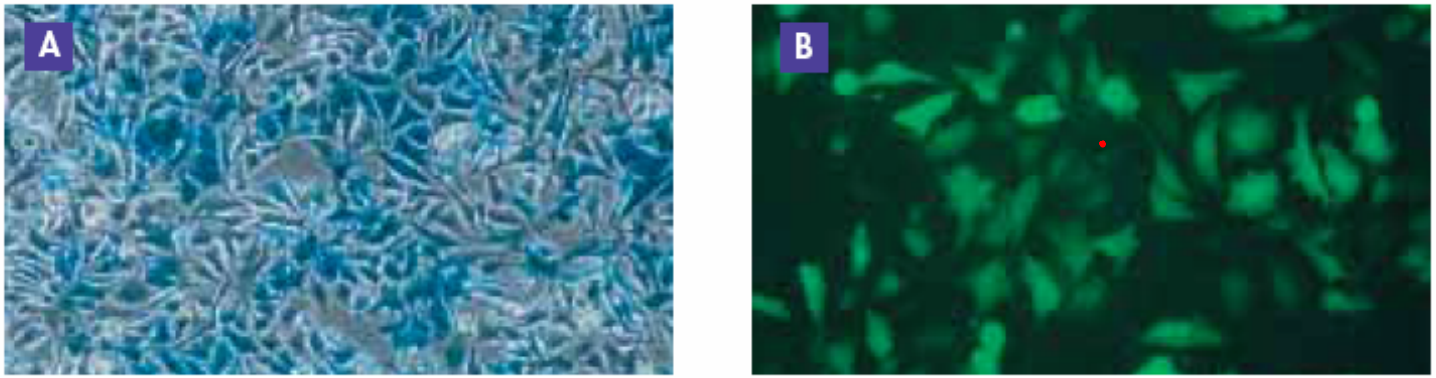
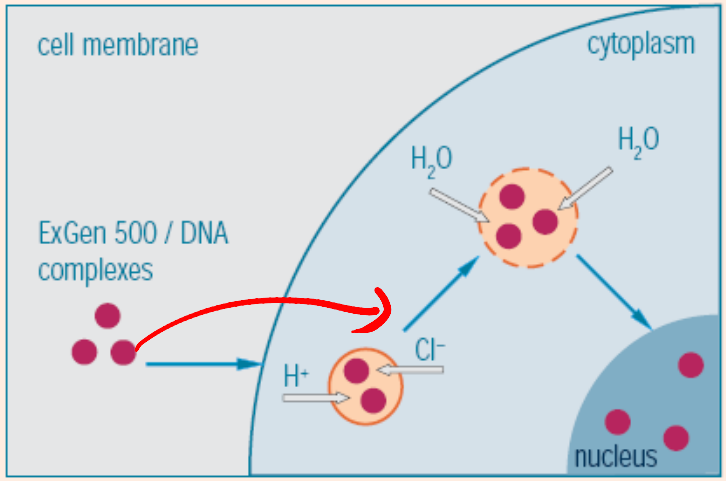
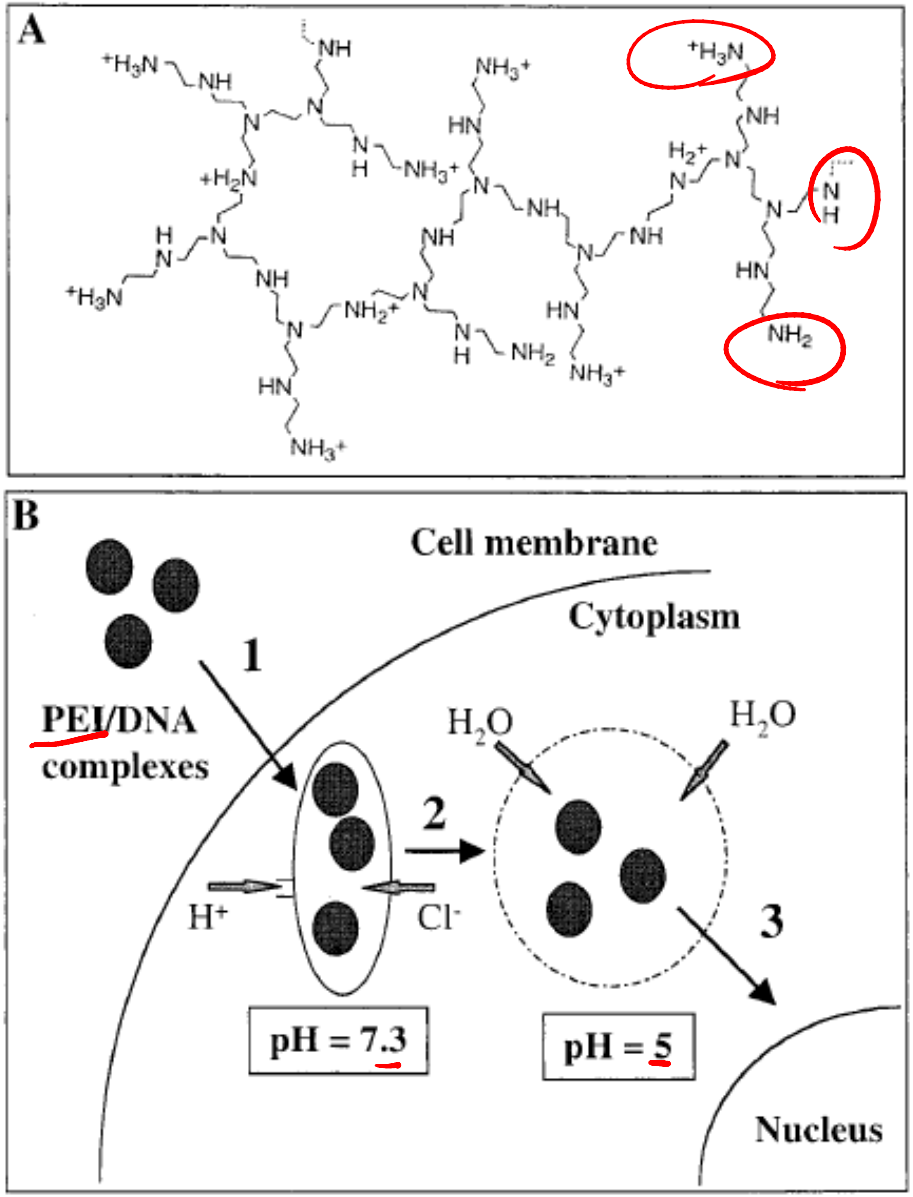


Figure 8. *Expression of* **A** *β-galactosidase and* **B** *green fluorescent protein (GFP) in HeLa cells. Cells were cotransfected in 6-well plates with β-galactosidase and GFP reporter plasmids using PolyFect Transfection Reagent and the HeLa cell protocol. Expression was visualized by X-gal staining or fluorescence microscopy 2 days post-transfection.*

Polymer



1. ExGen 500 interacts with DNA to form small, stable, highly diffusible complexes which are readily endocytosed.
2. "Proton-sponge" effect of ExGen 500 buffers endosomal pH by provoking massive proton accumulation and passive chloride influx.
3. Rapid osmotic swelling causes endosomal rupture, allowing translocation of DNA to the nucleus without DNA degradation.



PEI

Figure 1. (A) Structure of PEI with a random topology. (B) The 'proton sponge effect': after endocytosis of the cationic complexes (1), acidic endosome buffering (2) leads to increased osmotic pressure and finally to lysis (3)

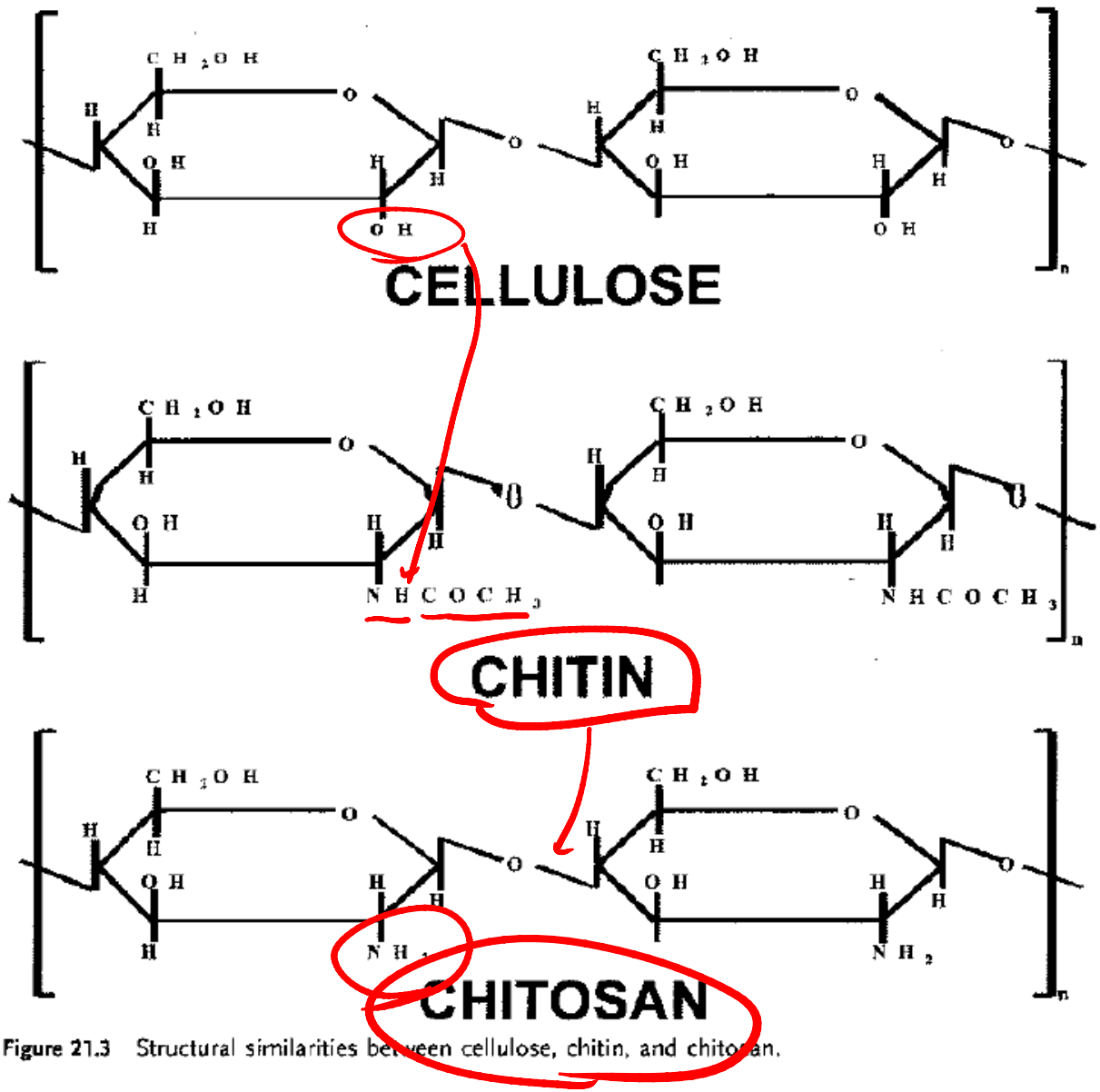
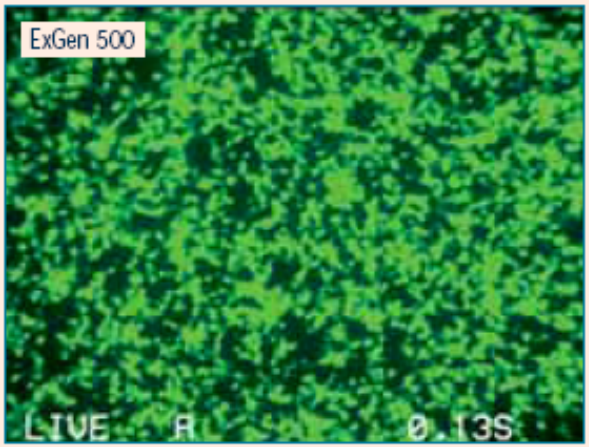
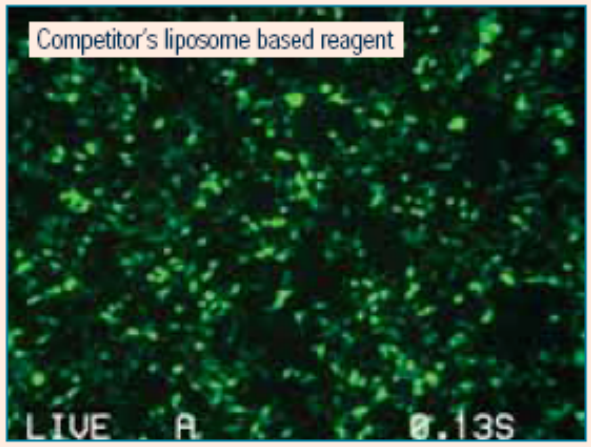


Figure 21.3 Structural similarities between cellulose, chitin, and chitosan.

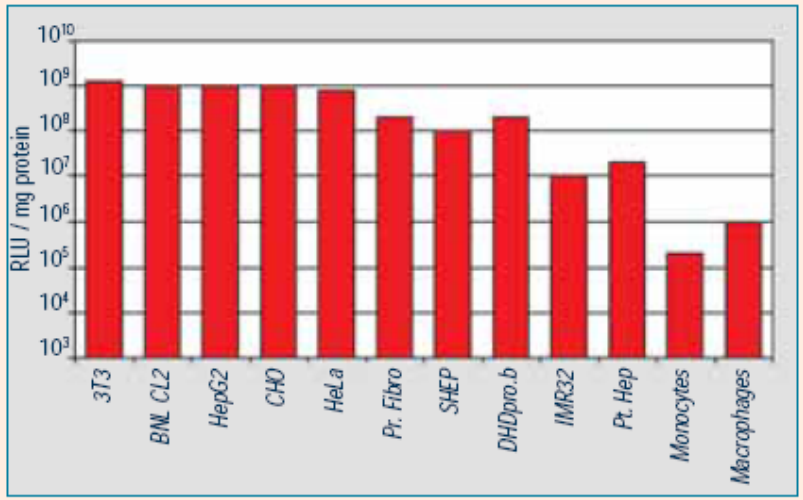
ExGen 500 performs when other transfection reagents fail





Expression of Green Fluorescent Protein (GFP) in 293 cells.
Cells were transfected with a vector containing the GFP coding sequence using ExGen 500 and competitor's liposome based reagent.

ExGen 500 transfects a wide variety of cell types



lipo ⇒
dendrimère
PEI

Gold Nanoparticles

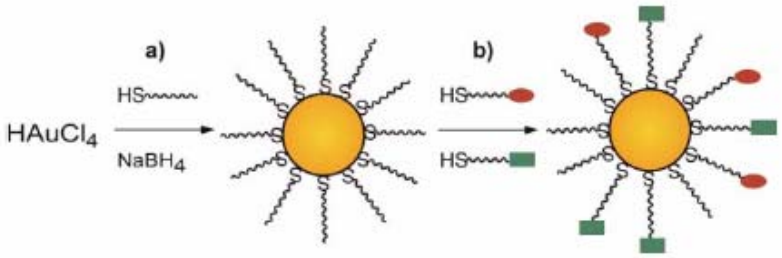
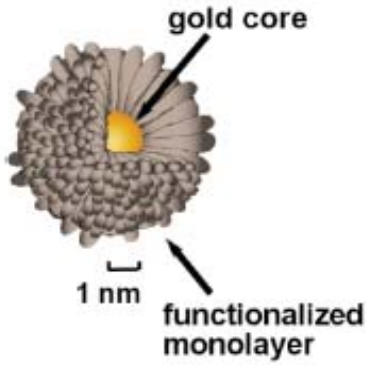
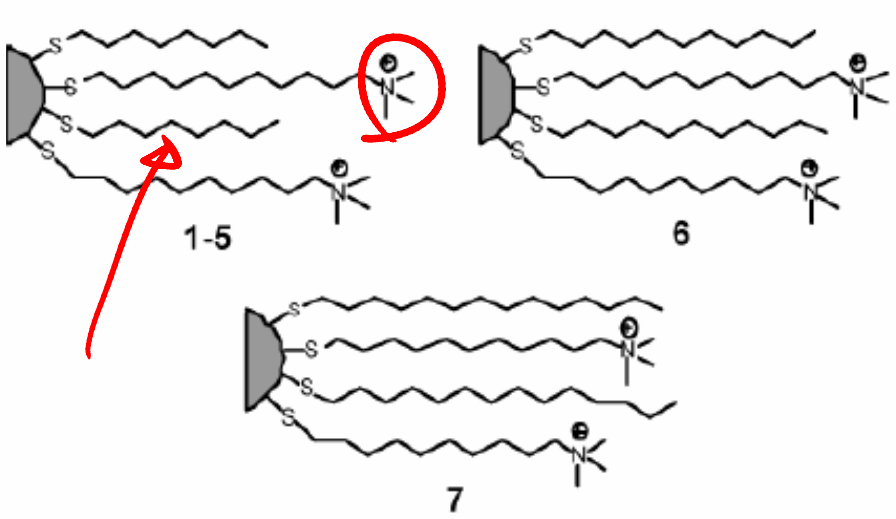


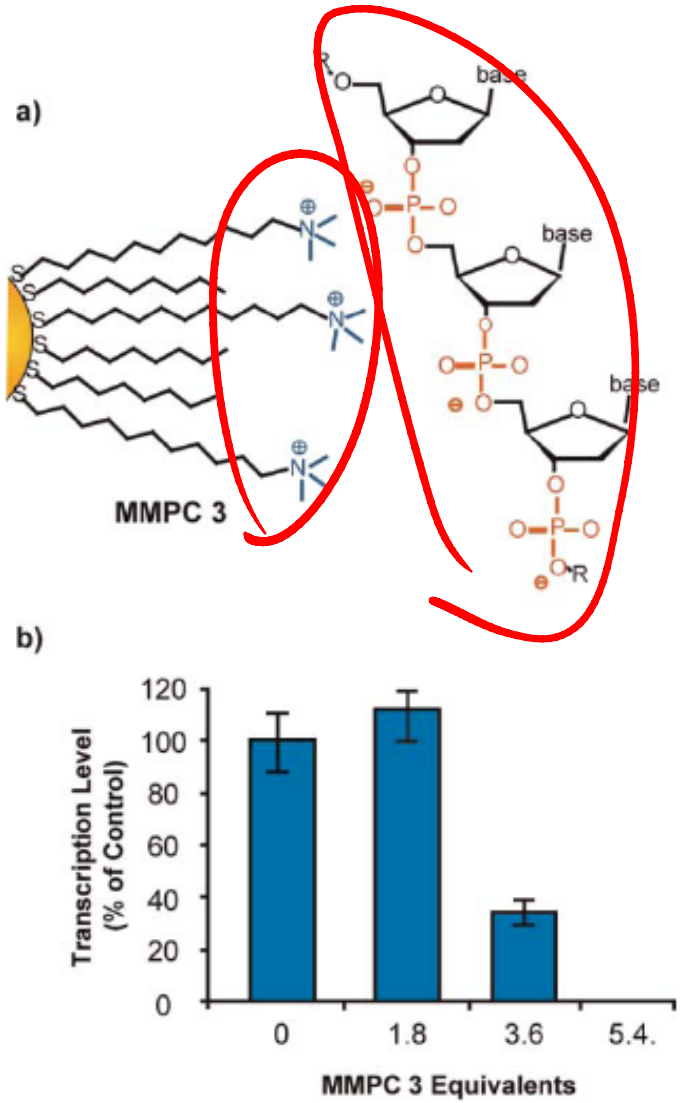
Fig. 2 Synthesis of gold MPCs using (a) the Brust–Schiffrin reaction and MMPCs *via* (b) the Murray place-exchange method.





MMPC	1	2	3	4	5	6	7
% cationic coverage	100	85	68	63	58	77	89

Figure 1. MMPCs used for transfection.



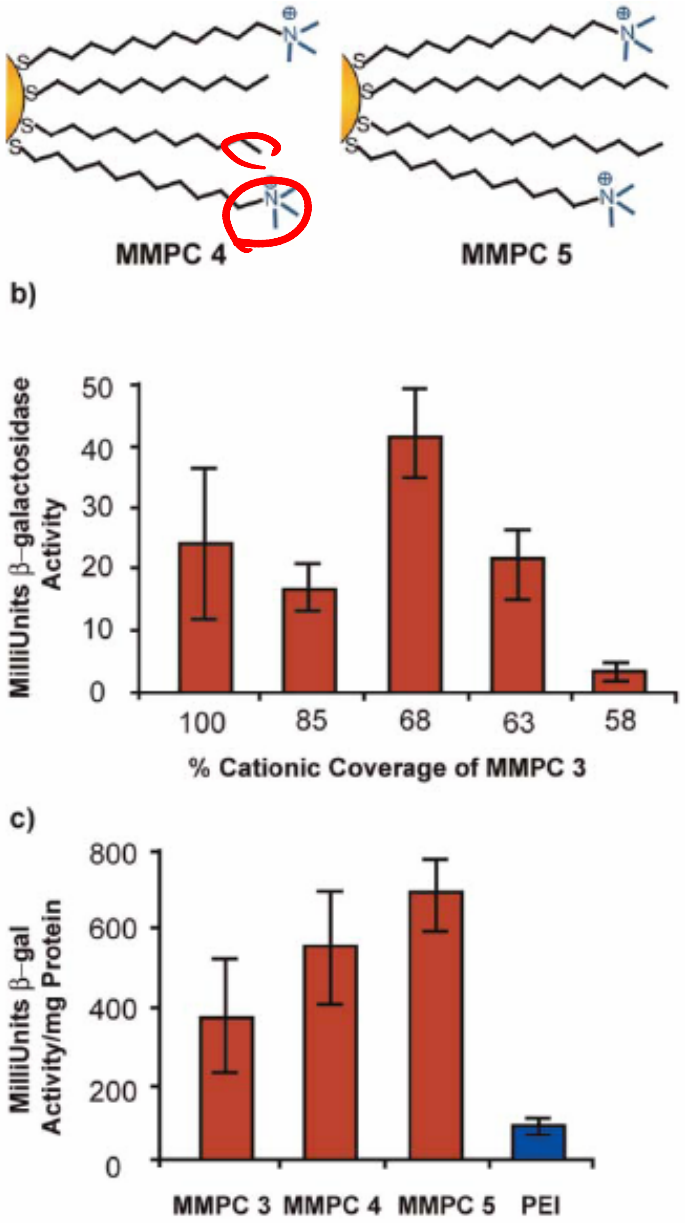
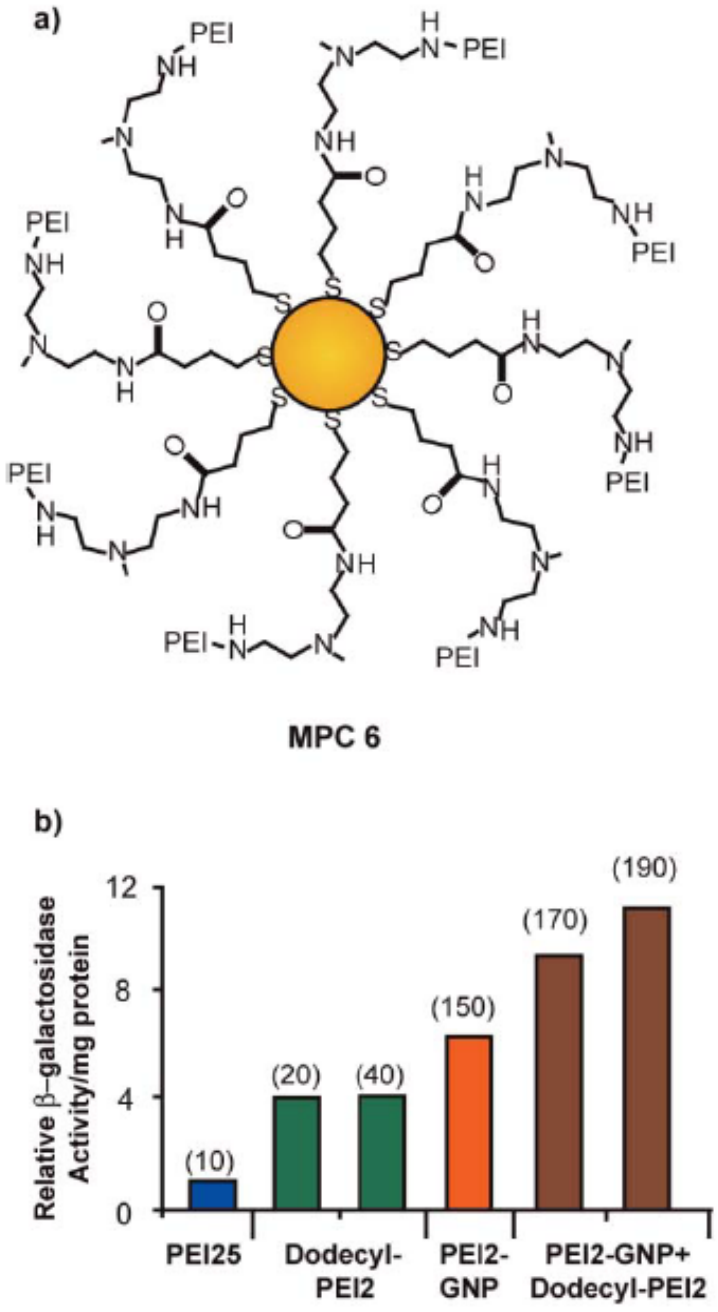


Fig. 5 (a) Structure of MMPC **3** scaffold and the DNA backbone. (b) Percent transcription level decreases with the increase in the amount of MMPC **3**.


a)
PEI
MPC 6
b)
Relative β–galactosidase Activity/mg protein
12
8
4
0
(10)
(20)
(40)
(150)
(170)
(190)
PEI25
Dodecyl-PEI2
PEI2-GNP
PEI2-GNP+ Dodecyl-PEI2

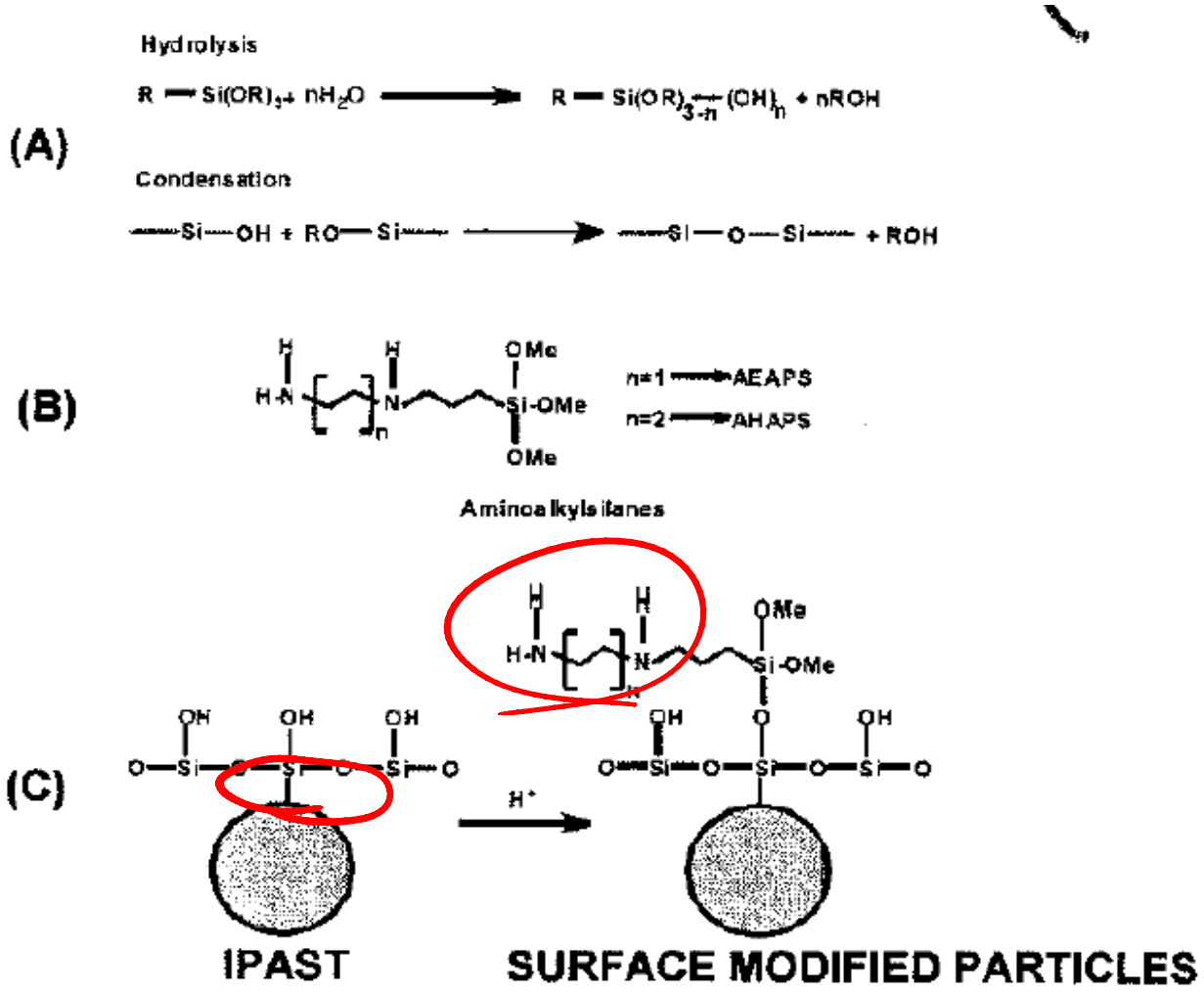
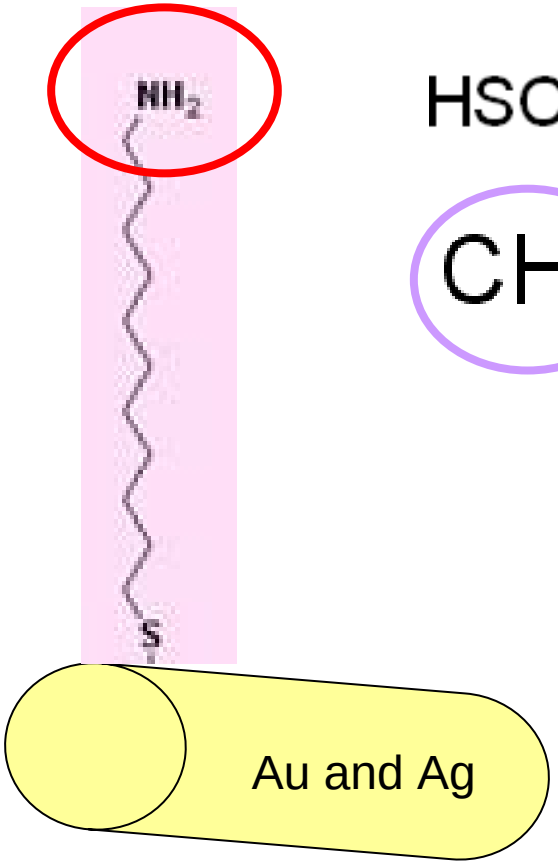
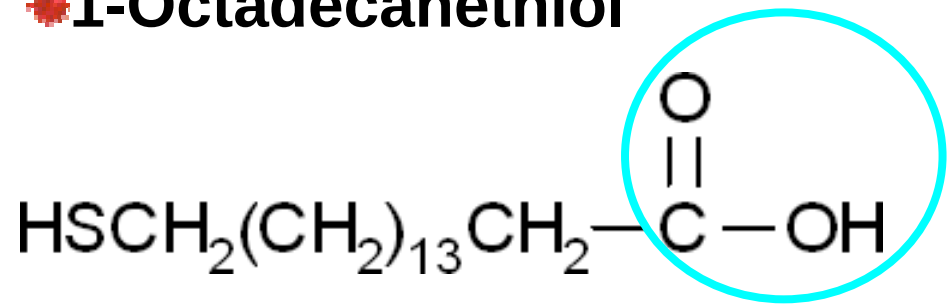


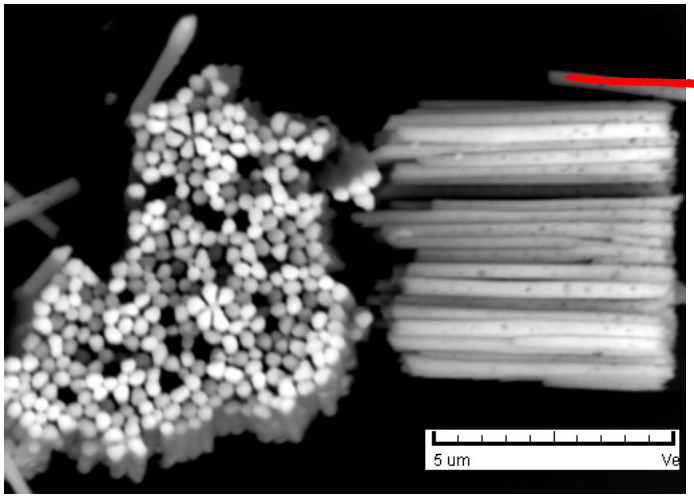
Figure 21.11 (A) Hydrolysis and condensation of unmodified particles. (B) Alkylaminoalkanes. (C) Modification scheme.

- 11-Amino-1-undecanethiol
- 16-Mercaptohexadecanoic acid
- 1-Octadecanethiol



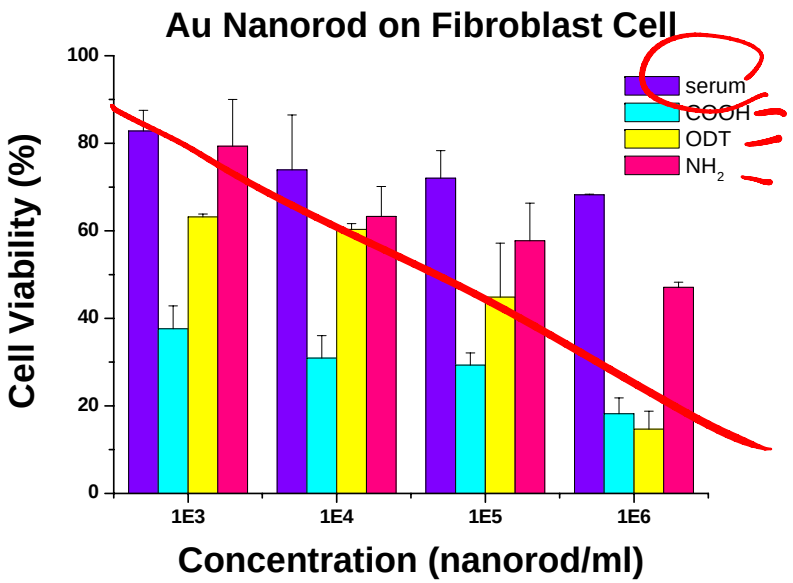


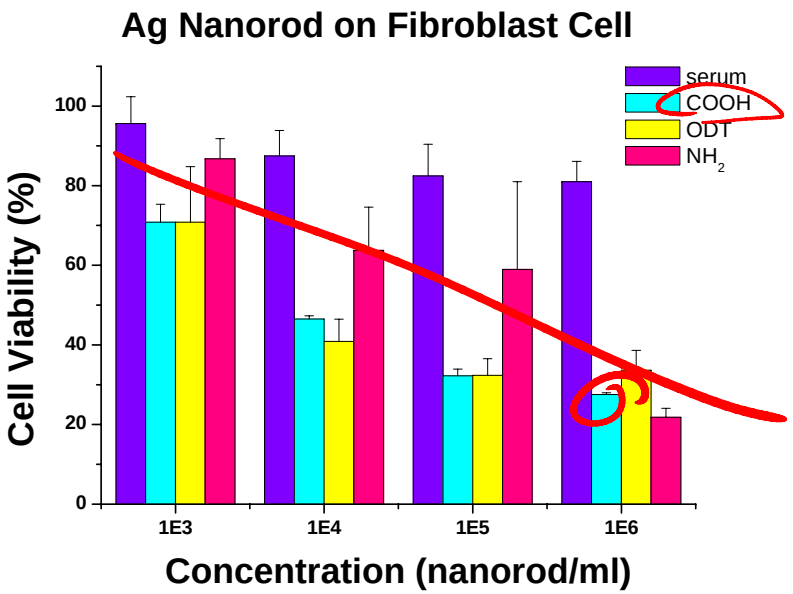
$CH_3(CH_2)_{16}CH_2SH$

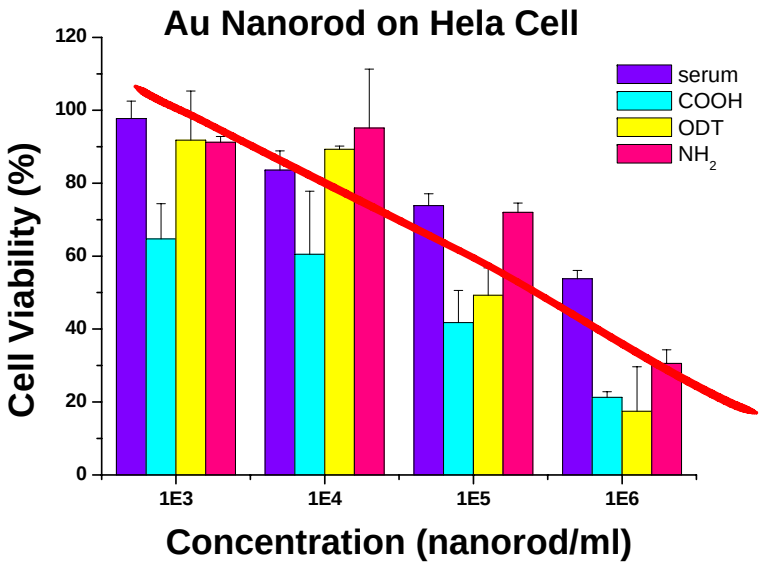


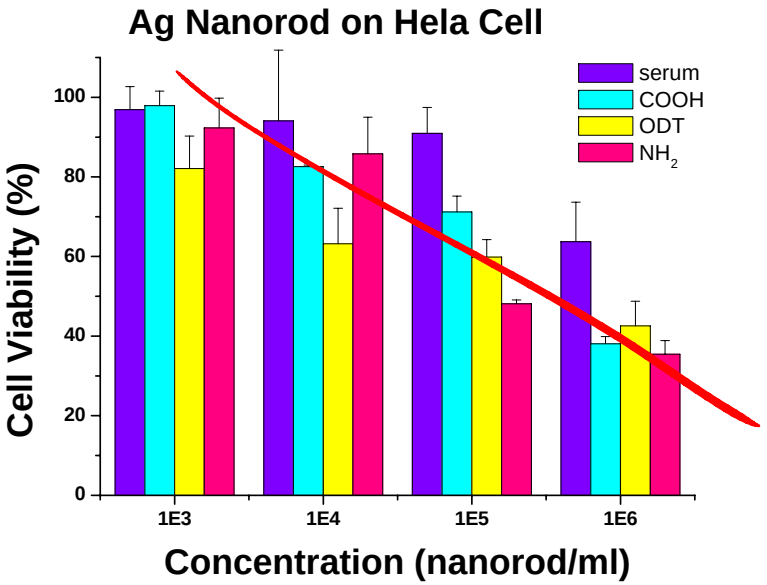
Au

Ag

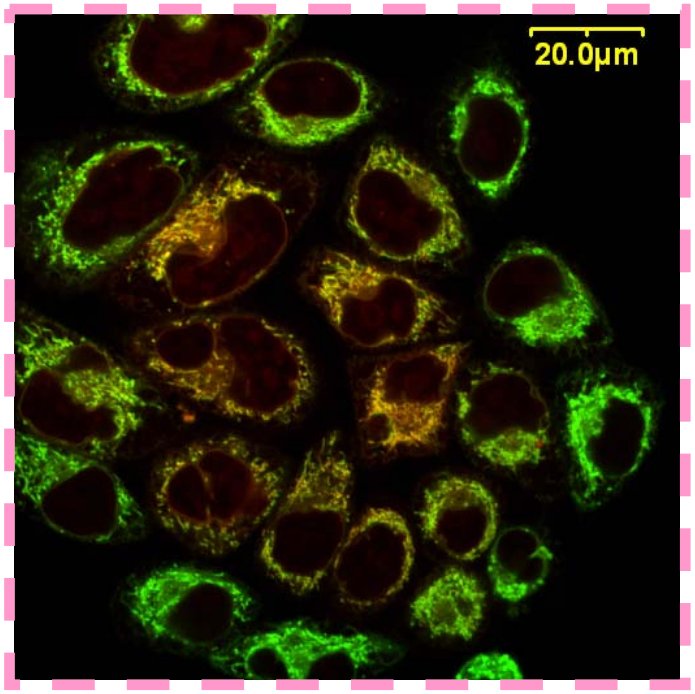

Au Nanorod on Fibroblast Cell
Cell Viability (%)
Concentration (nanorod/ml)
serum
COOH
ODT
NH2
0
20
40
60
80
100
1E3
1E4
1E5
1E6

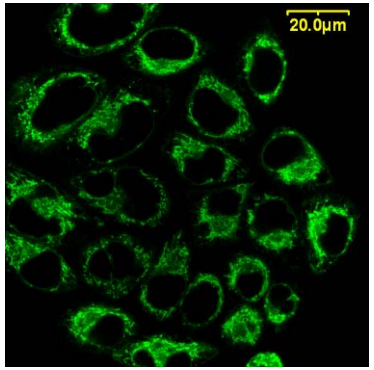

Ag Nanorod on Fibroblast Cell
Cell Viability (%)
Concentration (nanorod/ml)
serum
COOH
ODT
NH2
0
20
40
60
80
100
1E3
1E4
1E5
1E6

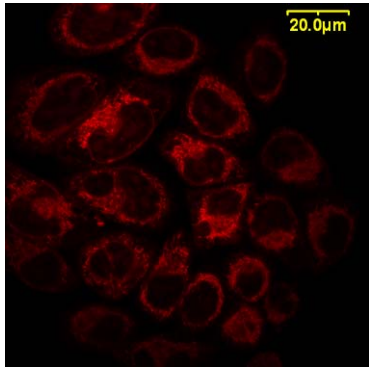

Au Nanorod on Hela Cell
Cell Viability (%)
Concentration (nanorod/ml)
serum
COOH
ODT
NH2
0
20
40
60
80
100
120
1E3
1E4
1E5
1E6

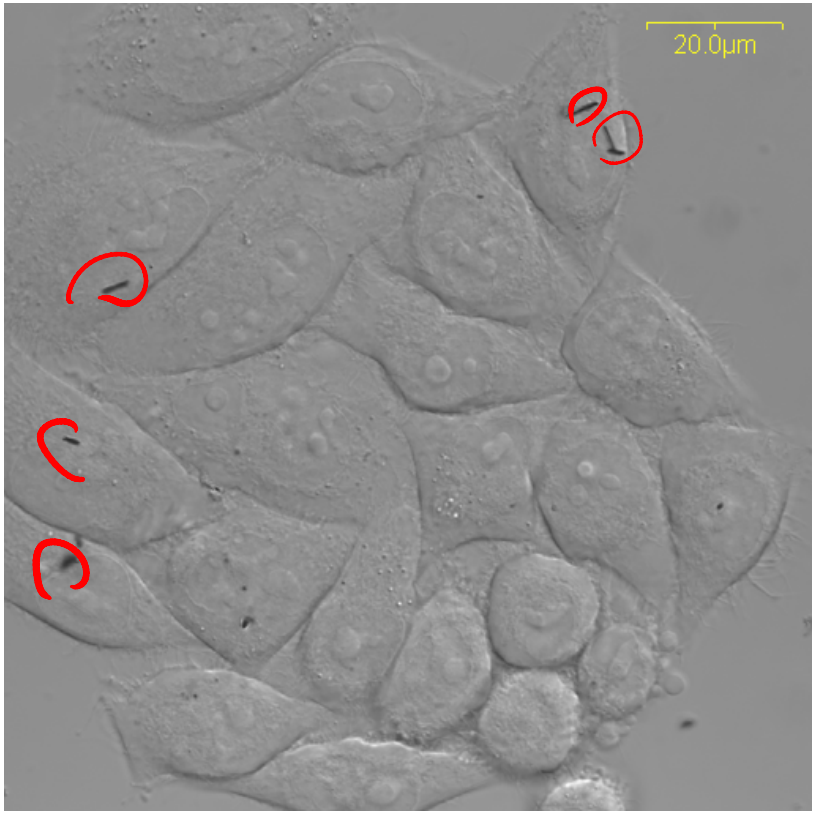

Ag Nanorod on Hela Cell
Cell Viability (%)
Concentration (nanorod/ml)
serum
COOH
ODT
NH2
0
20
40
60
80
100
1E3
1E4
1E5
1E6

HeLa cells with Au serum nanorods



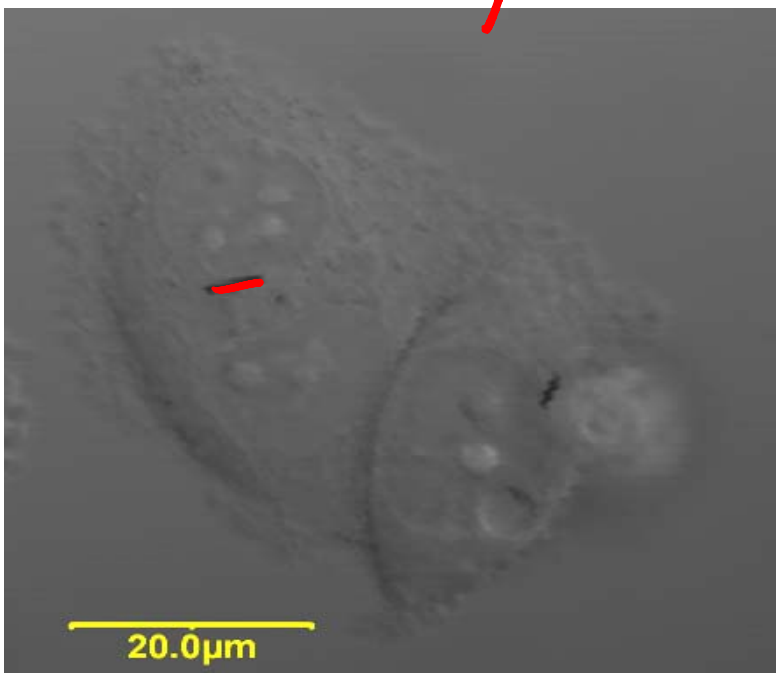


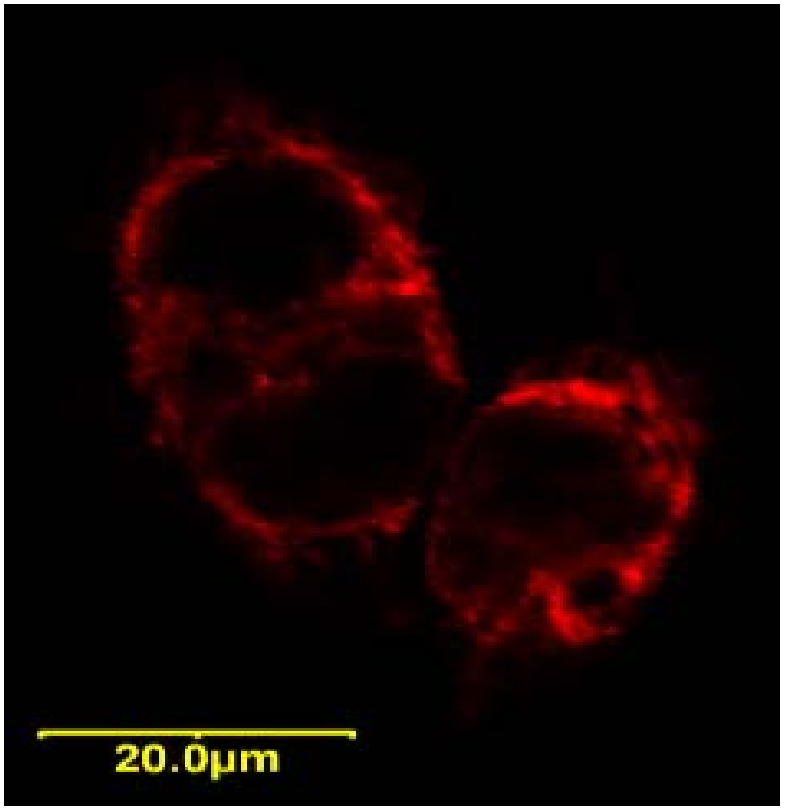




60X by Mito and LDS751 staining 0216 (conc. 5E4)

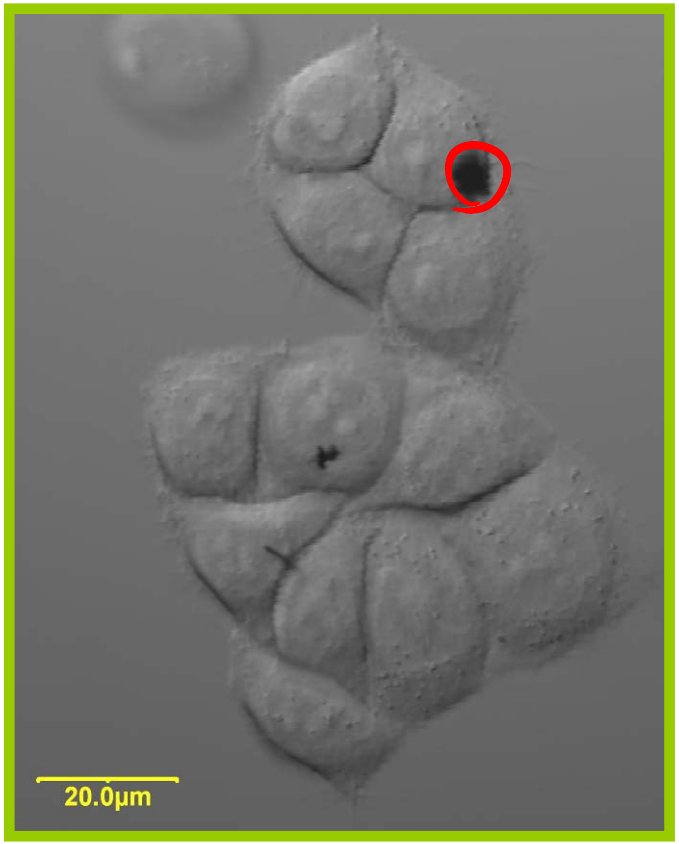
HeLa cell incubated with Au-NH_2 nanorods for 24h

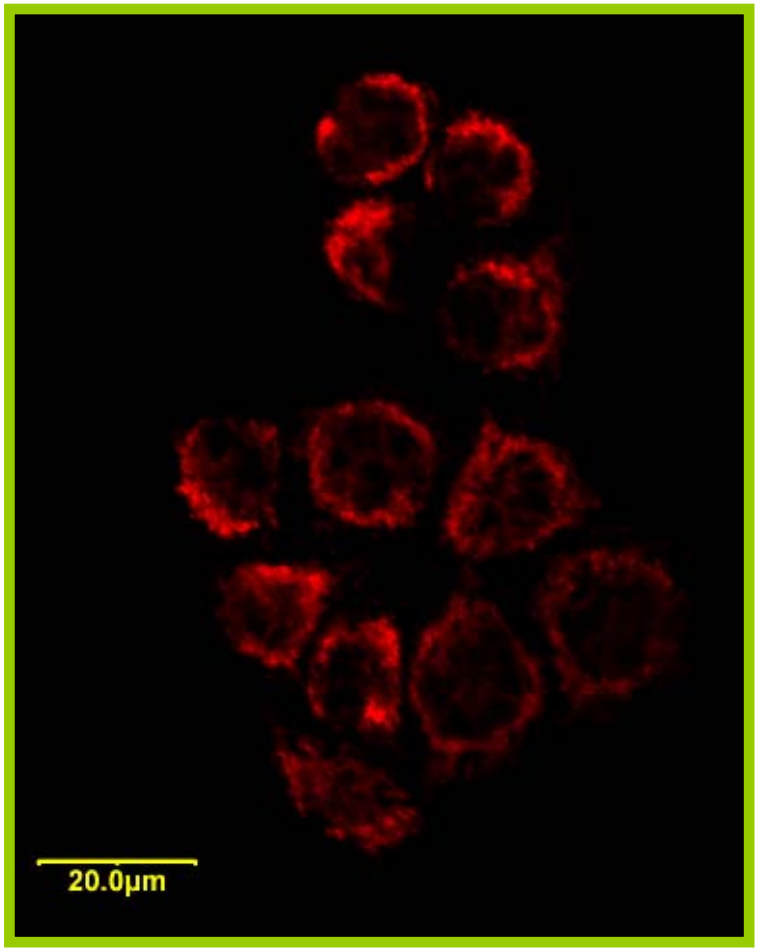




60X by LDS-751
staining 17012006

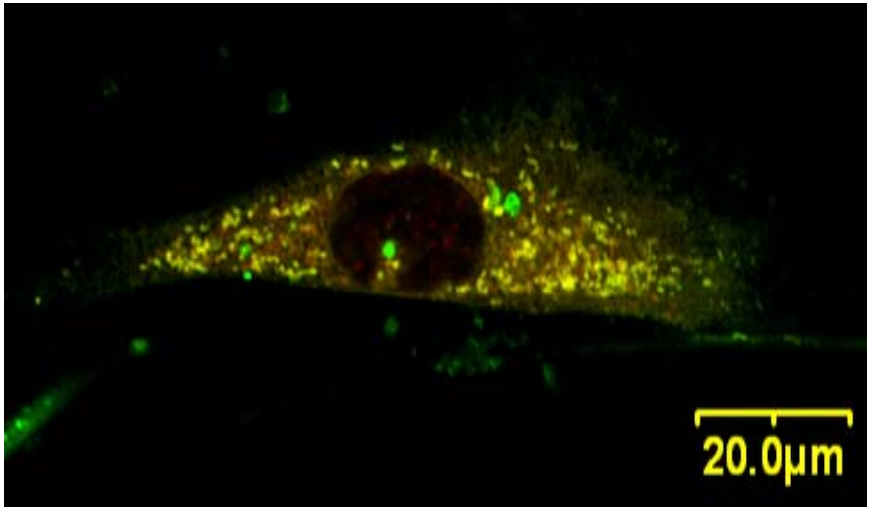
HeLa cell incubated with Au-ODT nanorods for 24h

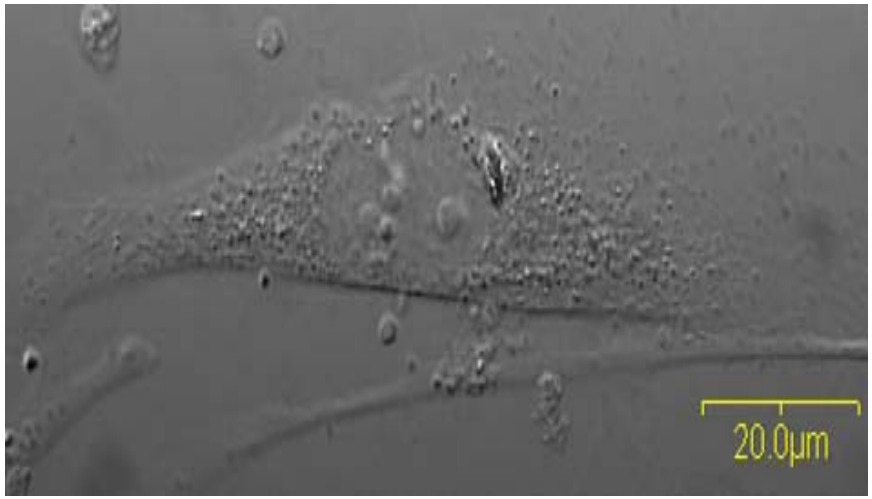


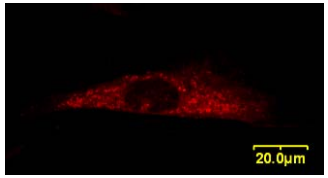


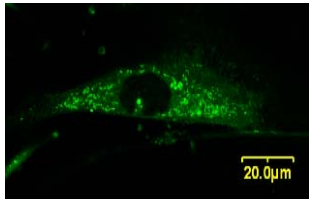
60X

Fibroblast cell incubated with Au-COOH nanorods for 24h



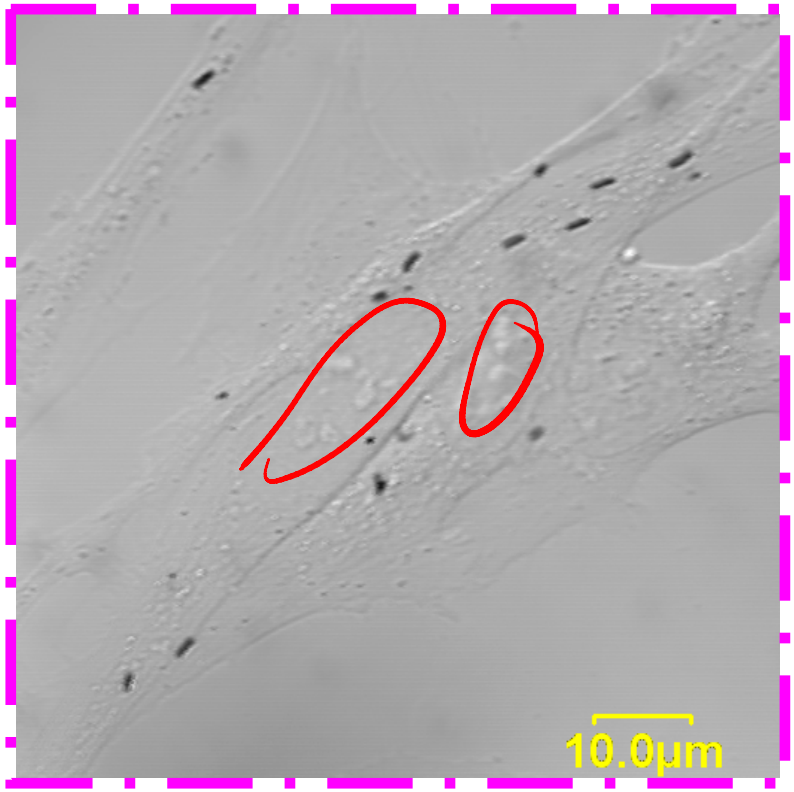


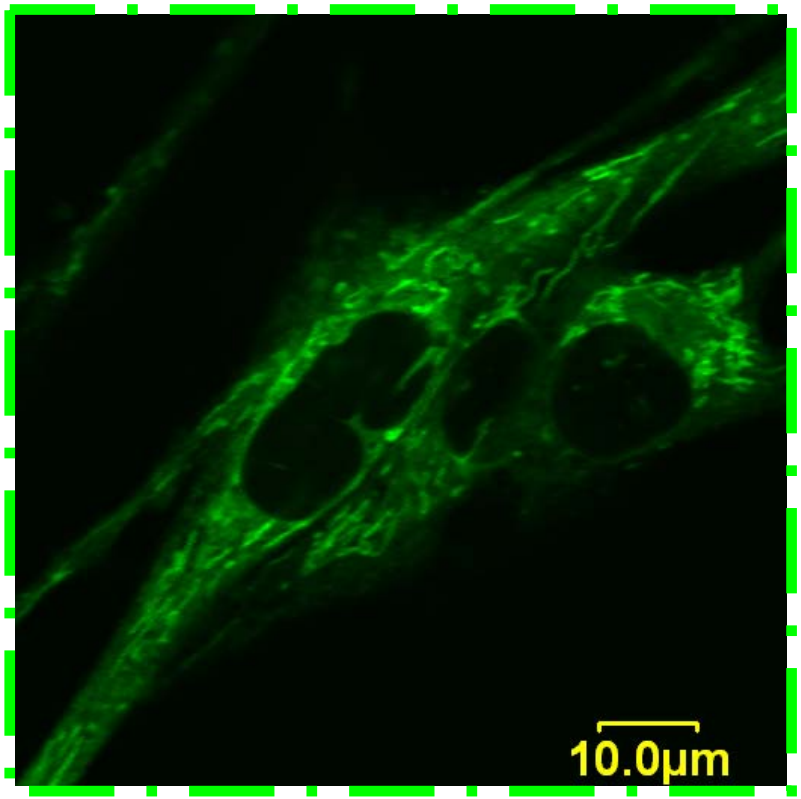




60X by Mito and LDS751 staining 0222 (conc.4E4)

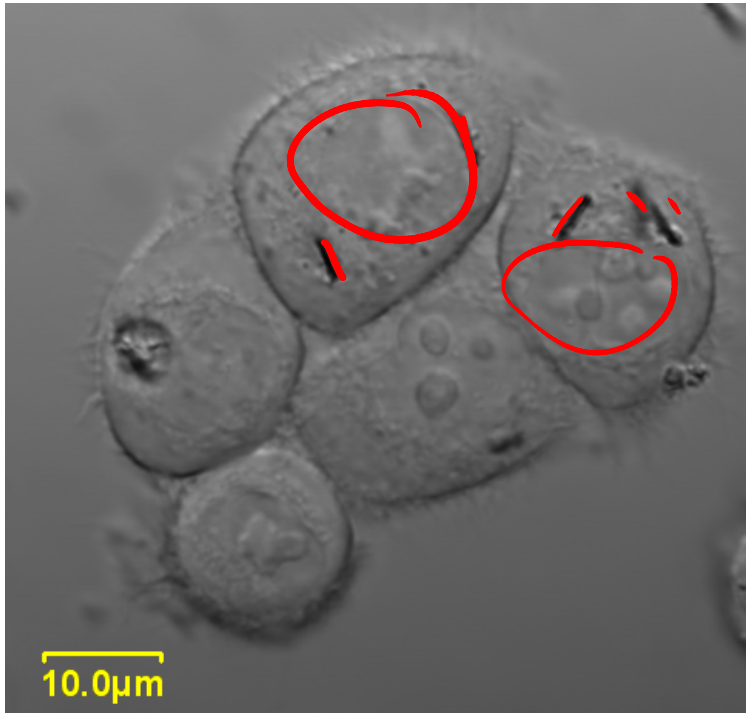
Fibroblast cell incubated with Ag-serum nanorods for 24h

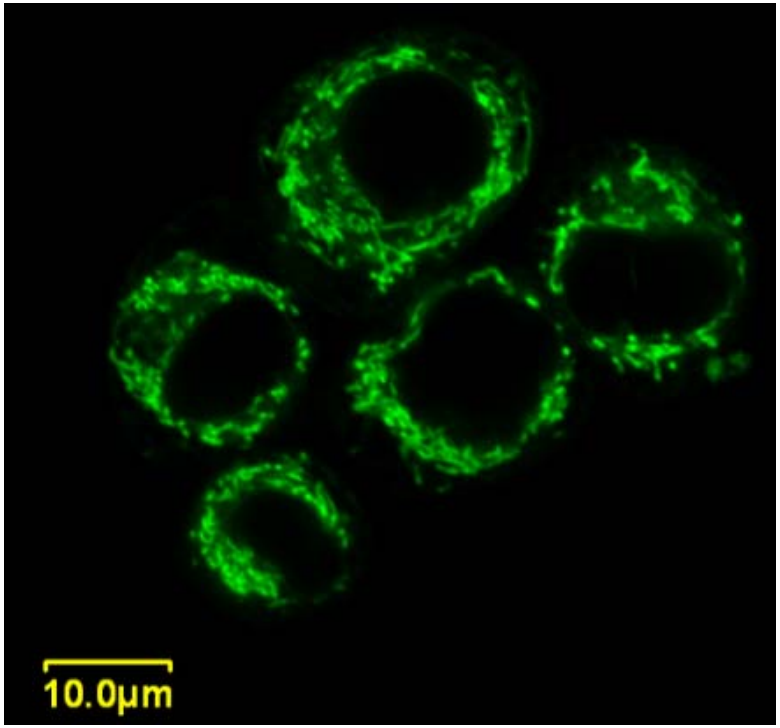




60X by Mito staining
0124 (conc. 2.5E4)

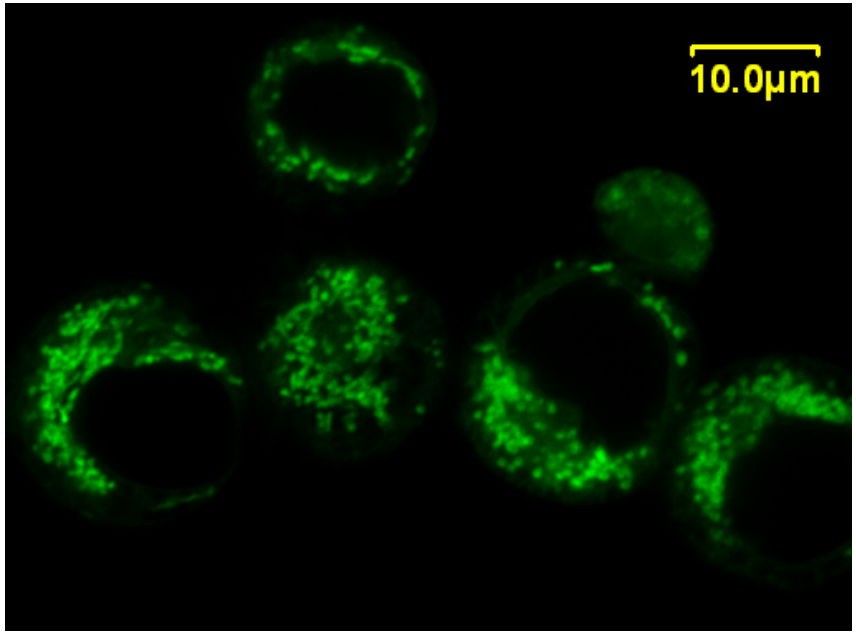
HeLa cell incubated with Ag-COOH nanorods for 24h





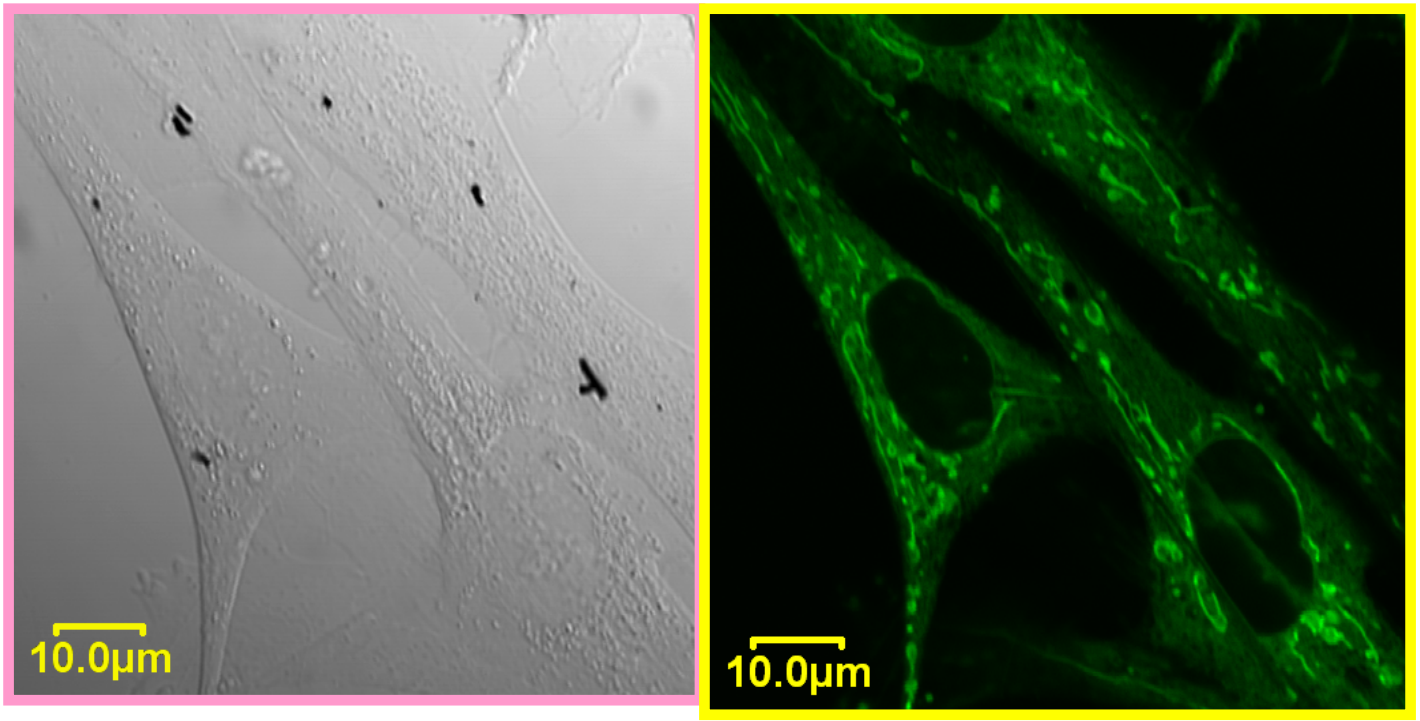
60X by Mito staining
0222 (conc.4.6E4)

HeLa cell incubated with Ag-ODT nanorods for 24 h



60X by Mito staining
0222 (conc.4E4)

Fibroblast cell incubated with Ag-NH_2 nanorods for 24h



60X- by Mito staining 0124 (conc. 3E4)

Parameters

- Cell density
- Amount of DNA
- Transfection reagent to DNA ratio
- Incubation period with DNA complex
- Incubation time following transfection

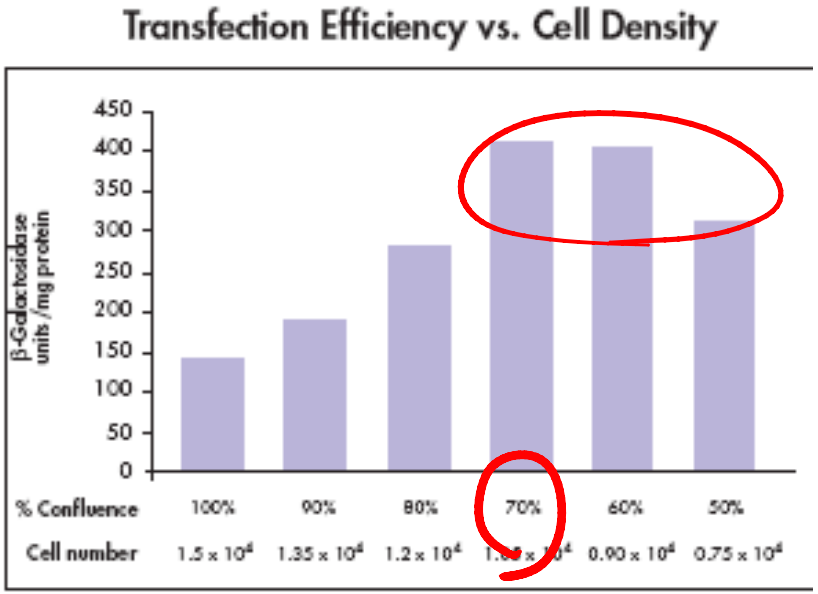


Figure 16. Transfections were performed in 96-well forma using 0.1 µg of a β-galactosidase reporter plasmid, 0.8 µ Enhancer, and 1 µl Effectene Reagent per well. Th indicated numbers of HeLa-S3 cells were seeded one da prior to transfection to provide cell densities o approximately 50–100% at the time of transfectior Transfection efficiencies and protein content wer measured from four replicates 48 h after transfection.

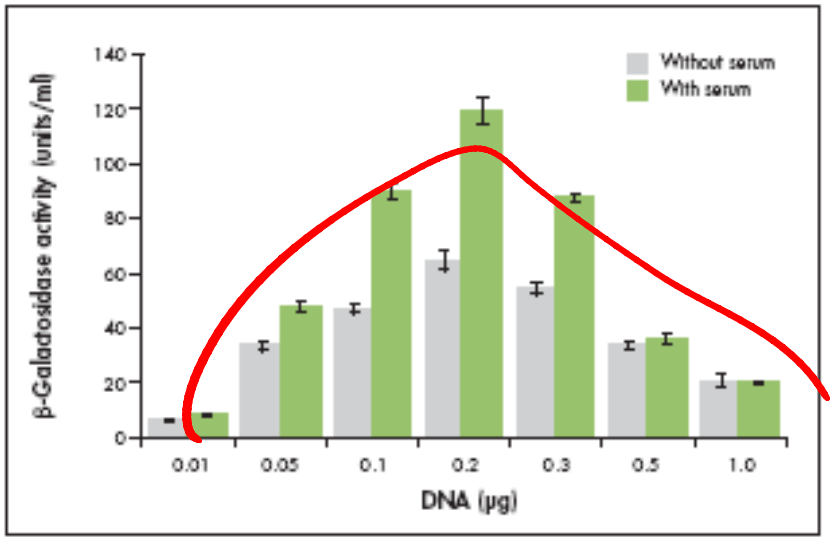


Figure 13. Influence of serum and DNA quantity on transfection using Effectene Reagent. 2 x 10⁴ COS-7 cells were seeded per well in 96-well plates one day before transfection. Cells were transfected using 0.01–1.0 µg of a β–galactosidase reporter plasmid and 0.08–8.0 µl Enhancer (DNA: Enhancer ratio of 1:8) and 2 µl Effectene Reagent, in either the presence or absence of serum. Each bar represents the average efficiency from four replicates 48 h post-transfection.