

BBL747 Bionanotechnology (Semester II: 2022-2023)

I Lecture Schedule (in hours):

| | |
|--|-----------|
| Introduction | 1 |
| Self-assembly of biomolecules in nanotechnology; Bacterial S-Layer | 4 |
| Biomimetic Ferritin | 3 |
| Magnetosomes | 3 |
| Microbial Nanoparticle Production | 2 |
| Bacteriorhodopsin and its technical applications | 4 |
| Molecular imprinting | 2 |
| Polymer Nanocontainers | 4 |
| Molecular Lego: Design for molecular actuators | 3 |
| Ion channels as molecular switches, Patch clamp technique | 2 |
| Viral Nanoelectronics | 3 |
| Protein based nanoelectronics | 3 |
| Carbon Nanotubes: Towards next generation biosensors | 3 |
| Techniques used in Bionanotechnology | 3 |
| Nanoanalytics: Fluorescent Quantum Dots for Biological Labeling , Nanoparticle Molecular Labels | 2 |
| Total Lecture (hours) | 42 |

II Suggested Reference:

Niemeyer CM and Mirkin CA. Nanobiotechnology: Concepts, applications and perspectives Wiley-VCH 2004

III Evaluation:

| | |
|------------|-----|
| Minor I | 20 |
| Minor II | 20 |
| Assignment | 15 |
| Seminar | 15 |
| Major | 30 |
| Total | 100 |

Audit Pass = 75% Attendance + Minimum 30% Marks

Self-Assembly

Materials that make themselves are beginning to make their presence felt as novel drug-delivery vehicles and electronic components

INTRODUCTION

Assembling molecules into complex structures:

- Natural
- Man-Made (Materials Scientists)

For example, chemists and clams. Both make high-strength ceramic composites.

- Chemists make use of crude methods-including extreme temperatures and the use of molds-to fuse neighboring molecules into specific shapes.
- The mollusks engineer a shiny, tough mother-of-pearl shell by using a series of proteins that assemble themselves into a scaffolding.
- The scaffolding guides tiny ceramic plates, created by the mollusk, into precise shell layers.

INTRODUCTION

- Biological organisms can organize structures by themselves at the angstrom, micron, and centimeter level.
- Materials scientists are increasingly attempting to use such materials that make themselves, being inspired from nature.
- The man made self assembled structures are still at the beginning stages and is growing up fast.
- Although scientists are no-where near duplicating the nature's more elegant self-assemblies but they have already begun to register their first practical success.

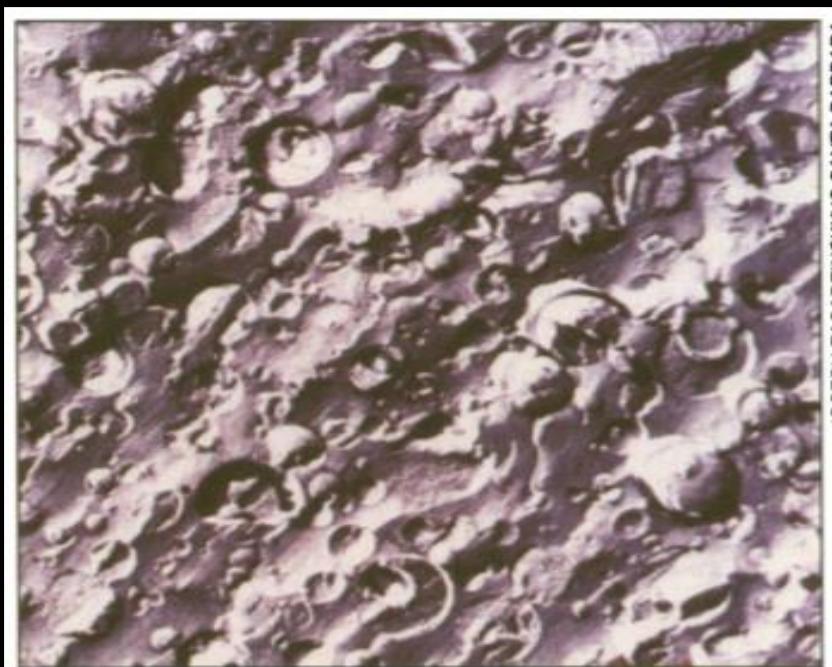
INTRODUCTION

- Several drug companies are in late-stage clinical trials with self-assembled microscopic vesicles that can carry potentially life saving drugs to cancer patients.
- And by the use of organic, metal, and phosphonate molecules (complexes of phosphorus and oxygen atoms derived from phosphonic acid H_3PO_3), to assemble themselves into conducting materials, researchers are turning electronic fabrication into a benchtop affair.

Applications:

1) Delivery Molecules

- Today, self-assembly appears to be coming together most quickly in drug delivery.
- But, medical shuttles must ward off the destructive attacks set in motion by metabolic processes.



Molecular shuttles. Self-assembling spheres such as these encapsulate anti-cancer chemotherapy agents and are currently in clinical trials.

Applications:

Drug Carriers

- Self-assembly gives researchers a tool to quickly and easily manufacture microscopic structures
- to protect these drugs in a hope that they can remain active in the body for longer periods, from days to weeks.
- As early as the 1960s, researchers realized that one possible way to sneak drugs into the body would be to hide them in a material imitating the membranes that make up the body's own cells and vesicles.
- These membranes are largely made of phospholipid molecules, which have balloon-like head groups that are attracted to water, and tails made up of long hydrocarbon chains that flee from water's presence.

Applications:

Drug Carriers

- By dissolving therapeutic drugs in the water at the start of this process, researchers were able to use these "lipid bilayers" as drug carriers, which can vary in size from 25 nanometers to more than a micron.
- The drugs were released either by slowly leaking out through the porous membranes or all at once when the bilayers ruptured.

Applications:

Trial, error, success

- Even though the idea of spheres carrying anti-cancer drugs was appealing, and were heavily hyped as a way to beat the disease, early versions of these self-assembling drug carriers had problems of being attacked by immune cells called macrophages.
- The mechanism of recognition by the immune cells isn't clear.
- It is most likely due to proteins in blood plasma that attached themselves to the spheres, tagging them for removal by macrophages.

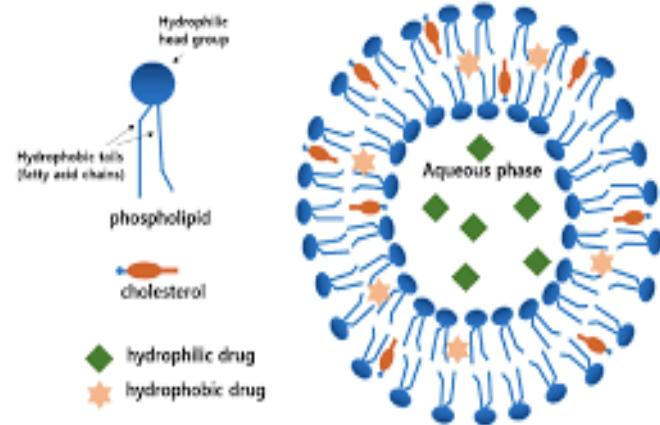
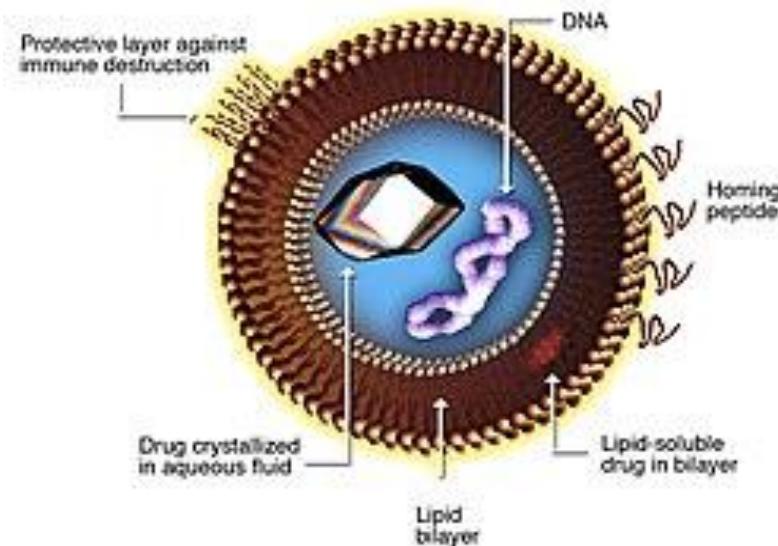
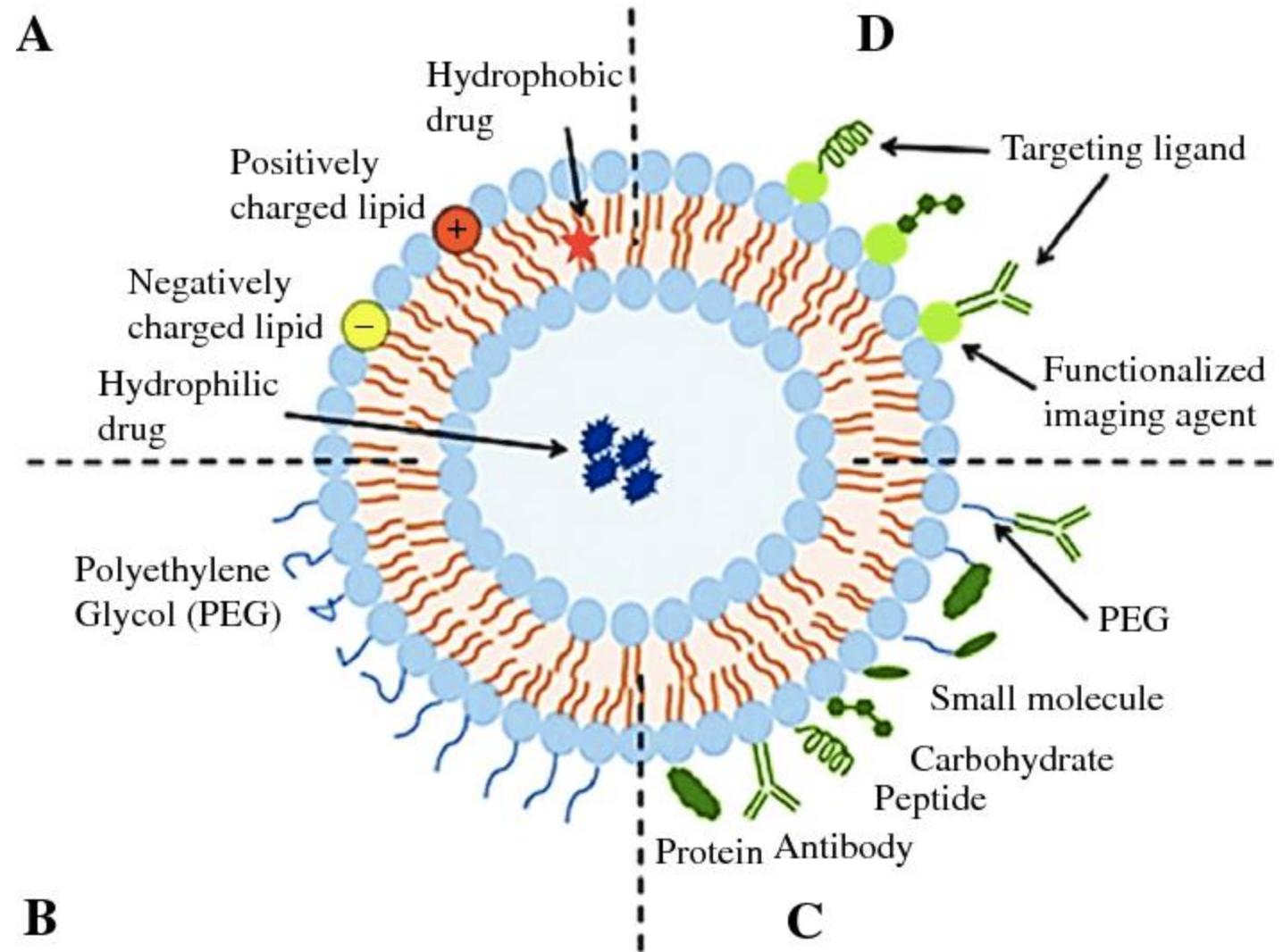


Figure 3. Structure of conventional liposome encapsulating hydrophilic and hydrophobic drugs.

Liposome for Drug Delivery





Applications:

Trial, error, success

- Natural membranes and vesicles escape this fate because they incorporate other molecules, such as glycoproteins and carbohydrates. (RBCs life span is 120 days)
- These modifications stick out of the membrane and apparently help shield them from attachment by proteins.

Applications:

Trial, error, success

- In the late 1980s, when two independent groups, led by **Demetrios Papahadjopoulos**, a biophysicist at the University of California at San Francisco, and **Terry Allen** at the University of Alberta in Edmonton, began mimicking the protection that natural membranes use by incorporating glycolipids into the liposomes.
- It worked: In animal tests the liposomes escaped assault by macrophages.
- In the last few years academic and biotechnology company researchers have pulled off the same trick using synthetic glycolipids such as polyethyleneglycol (PEG).

Applications:

Trial, error, success

- Based on that and other successes, liposomes are now being employed as transport vehicles for several different types of drugs.
- In early trials on animals, two companies Liposome Technologies of Menlo Park, California, and The Liposome Company of Princeton, New Jersey have shown that encasing the anti-cancer drug doxorubicin in liposomes and injecting it into cancer stricken mice can increase survival rates of the mice over others that receive the drug all by itself.

Applications:

Further Enhancements

- Liposomes now look promising for drug delivery, and are being further modified to specifically target a tumor and deliver their cargo just to the tumor cells.
- And that approach called as "targeted spheres" for targeted delivery.

Example: attachment of an antibody for mammalian squamous-cell carcinoma to a sphere that successfully attacked lung tumor cells in mice.

- Robert Langer at the Massachusetts Institute of Technology tried to self-assemble drug-carrying spheres out of polymers, some of which may break down more slowly, thereby releasing their contents over a longer period of time.
- Increased Shelf- Life.

Self Assembly- Applications:

2) The Tubular Route

- Spheres aren't the only structures that phospholipids can form.
- Lipids other than PC have features that cause them to assemble into tubes that resemble microscopic soda straws.
- And the ability of these "microtubules" to form spontaneously allows researchers to explore materials that would be less time consuming and expensive.



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Drawing straws. Used for applications ranging from drug delivery to microwave electronics, strawlike "microtubules" can be as small as half a micron in diameter.

Applications:

2) The Tubular Route

- Tubule-forming phospholipids such as diacetylenic lipids have a slightly different shape than sphere-forming phospholipids like PC.
- NRL's Schnur with his colleague Jonathan Selinger worked out a model of microtubule formation.
- According to this model, when these phospholipids begin stacking in bilayers, the molecules' asymmetrical shape keeps them from forming a sphere and forming a long, narrow strip.

Applications:

2) The Tubular Route

- The disjointed packing within the bilayer then causes each strip to curl, rather like a spiral staircase.
- Attraction between intermolecular forces at the edges winds the staircase tighter until the edges fuse to form a tubule.

Applications:

2) The Tubular Route

- Once formed, these tubules can then be stuffed with a Jello like polymer matrix containing drugs or other chemicals that will slowly drain out, as the polymer is porous.
- Further two biologists, Alan Rudolph and Barry Spargo used this strategy to turn microtubules into timed-release drug capsules.
- In animal studies, they showed that tubules stuffed with the polymer matrix and a cell growth factor called transforming growth factor, when placed at the site of a wound, would slowly release the growth factor for up to 5 days, speeding wound healing.

Applications:

2) The Tubular Route

- The microtubules also make dandy microscopic templates when coated with metals, they are stronger and have useful electronic properties.
- Physicists Ranganathan Shashidhar of NRL and Eric Cross of Pennsylvania State University explored the use of nickel and permalloy tubules to reduce the electromagnetic interference between adjacent microchips in ultra dense circuit boards.

Applications:

2) The Tubular Route

- Such microchips constantly give off and absorb electromagnetic waves, like radio waves and microwaves.
- If strong enough, the electromagnetic waves given off from one chip can disrupt the operation of its neighbors .
- The need to miniaturize components by packing the chips closer together boosts the interference between chips.
- Therefore, the method employed was placing small pieces of material between each chip to block the electromagnetic fields and microscopic rods of metal were ideal for this, given their long thin shape that enhances their ability to block electromagnetic waves.

Applications:

3) Sheets and sandwiches

- Electronics applications are also driving researchers to experiment with self-assembly as a way of layering sheets of materials, an arrangement that has myriad applications.
- Laying down successive single-atom-thick layers of semiconductor materials for devices such as laser diodes by molecular beam epitaxy machine to "spray paint" molecular layers is a costly process.
- Self-assembly has an advantage over this. The molecular building blocks are chosen so that they can only combine in a single-layered arrangement.
- Technique to layer electronically active materials for devices such as solar cells and light-emitting diodes (LEDs).

Applications:

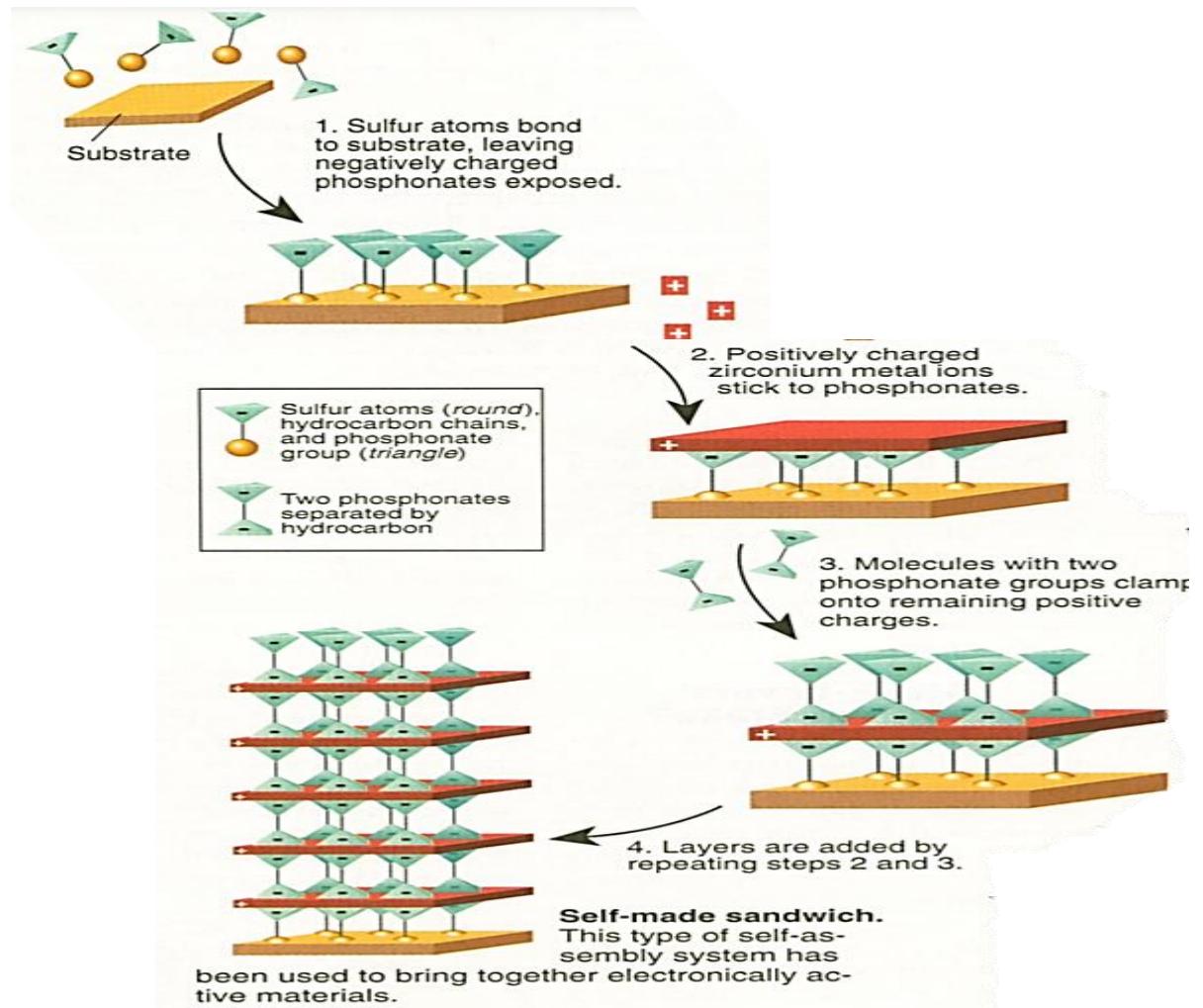
3) Sheets and sandwiches

- To make such self-assembled sandwiches in such a way that each layer serves as a surface for the next layer.
- In 1988, Mallouk first did this by layering a series of molecules on a gold-plated silicon bed.

Applications:

3) Sheets and sandwiches

- He started by dipping this base into a solution containing molecules with a sulfur atom at one end, a hydrocarbon chain in the middle, and a phosphonate group at the end .
- The sulfur atoms tightly bonded to the gold surface, leaving phosphonate groups sticking out at the surface.
- These phosphonates carry a net negative charge.
- Then the structures was placed in a bath containing zirconium ions, which have a positive charge.
- The zirconium bonded to the phosphonates, forming a single-atom thick layer of sheet metal.



- Then the structures was dipped in a bath containing molecules with phosphonate groups at both end, which readily bound to the zirconium, leaving another group of unbound phosphonates sticking out at the top.
- From there simply the process was repeated, building layer after layer.

Applications:

3) Sheets and sandwiches

- Layered structures are essential for semiconductor devices, which work by separating opposite electric charges between different layers, or different levels of the same layer.
- In conventional solar cells, silicon atoms capture photons, emitting negatively charged electrons and positively charged "holes".
- Effectively due to the absence of an electrons, the opposite charges migrate to the top and bottom of the silicon layer, where they are collected and shuttled to a battery for storage.
- If the charges recombine before reaching the battery, the result is heat instead of electricity.

Applications:

3) Sheets and sandwiches

- In 1992, Howard Katz and his colleagues at AT&T used Mallouk's "metalphosphonate" self-assembling system to emulate part of a solar cell.
- They began by depositing several layers of organic molecules, known as porphyrins and vilogens.
- Like silicon, porphyrin molecules capture sunlight, giving off electrons and holes.
- Here in the device the porphyrins donate the electrons to the vilogen molecules, allowing the charges to remain separate.

Applications:

3) Sheets and sandwiches

- self-assembled layers hold the promise of drastically reduce the manufacturing cost of solar cells.
- Further research is going on with self-constructing layers as possible routes not only to solar cells but to LEDs, which run the same reaction in reverse, combining opposite electric charges and emitting light.

Constructing the field

Further Applications:

- Researchers are trying to get self assembling molecules to aid in orienting liquid crystal polymers for optical displays.
- And, in making nonlinear optical materials, which are essential for routing optically transmitted data.

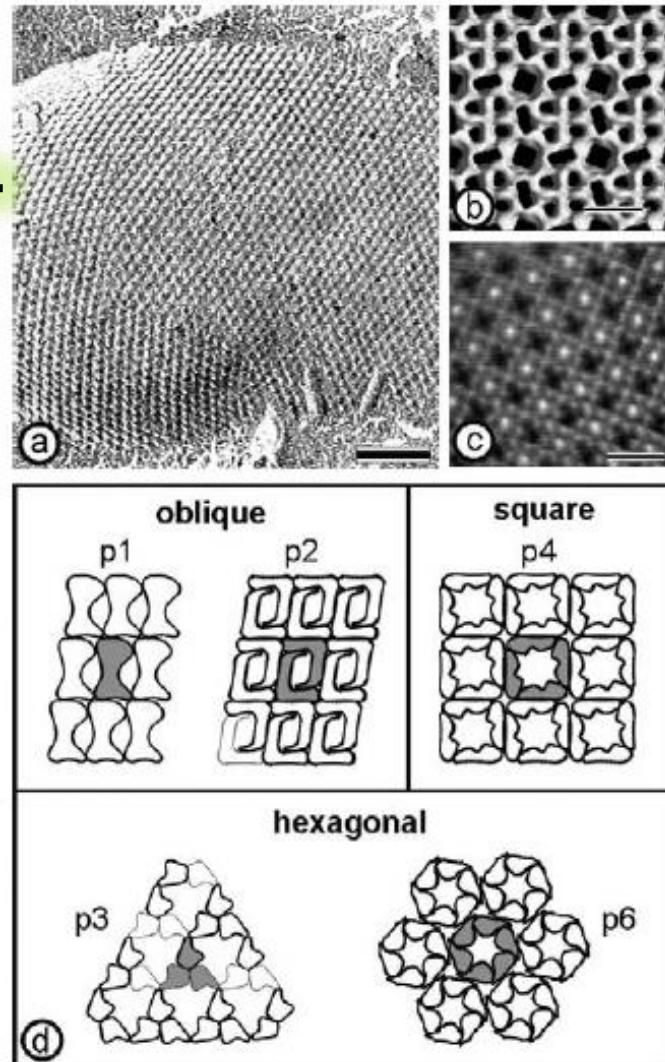
S-Layers

Introduction:

- Crystalline bacterial cell-surface layers (S-layers) have been optimized during billions of years of biological evolution as one of the **simplest biological membranes**
- S-layers are composed of a single protein or glycoprotein species endowed with the ability to assemble into monomolecular arrays on the **supporting cell envelope component** of prokaryotic organisms.
- The information accumulated on the structure, chemistry, assembly, genetics, and function of S-layers has led to a broad spectrum of applications for life and material sciences

Structure

Figure: (a) Freeze-etching preparation of whole cells of *Thermoanaerobacter thermohydrosulfuricus* L111-69 revealing a hexagonally ordered array. Scale bar = 100nm. (b) Three-dimensional model of the S-layer of *Bacillus stearothermophilus* NRS 2004/3a/V2 exhibiting oblique lattice symmetry. The protein meshwork shows one square shaped, two elongated, and four small pores per morphological unit. (c) Computer image reconstruction of scanning force microscopic images of the topography of the square S-layer lattice from *Bacillus sphaericus* CCM 2177. The images were taken under water. The surface corrugation corresponding to a gray scale tram black to white is 1.8 nm. Scale bars in (b) and (c) = 10 nm. (d) Schematic drawing of the different S-layer lattice types. The regular arrays exhibit either oblique (p1, p2), square (p4), or hexagonal lattice symmetry (p3, p6). The morphological units are composed of one, two, three, four, or six identical subunits. (Reproduced from Ref. [3], with permission from Wiley-VCH.)



Chemistry

- S-layers composed of single homogeneous protein or glycoprotein species
- Weakly acidic proteins ($\text{pI} = 4\text{--}6$), except *Methanothermus fervidus* ($\text{pI} = 8.4$) and *Lactobacilli* ($\text{pI} = 9.5$)
- Molecular weights ranging from 40 to 200 kDa.
- Post translational modifications in S-layer proteins, includes
 - Cleavage of amino- or carboxy-terminal fragments,
 - Phosphorylation
 - Glycosylation of amino acid residues.
- Glycosylation of amino acid residues is a characteristic of many archaeal and some bacterial S-layer proteins
- These glycan chains and linkages differ significantly from those of eukaryotes.

Structure

- In most S-layer proteins, ~ 20% of the amino acids are organized as α -helix, and about 40% occur as β -sheets
- The proteinaceous subunits of S-layers can be aligned in lattices with oblique, square, or hexagonal symmetry
- Center-to-center spacing of the morphological units of between 3 and 35 nm.
- S-layers are porous membranes, with pores occupying 30 to 70% of their surface area
- In both Gram-positive bacteria and archaea, the lattice assembles on the surface of the cell-wall matrix
- In Gram-negative bacteria the S-layer is attached to the outer membrane

Genetics and Secondary Cell-Wall

~~Polymers~~

- S-layer genes from organisms of different taxonomic affiliations have been cloned and sequenced.
- Considering the competitive situation of closely related organisms in their natural habitats, the S-layer surface must contribute **to diversification rather than to conservation**.
- Achieved by S-layer variation leading to the **expression of different types of S-layer genes, or to the recombination of partial coding sequences**.
- S-layer variation studied in *Campylobacter fetus*, an important pathogen for humans and ungulates but was also observed for nonpathogens such as *Geobacillus stearothermophilus*
- **High sequence identities are limited to the N-terminal region**

Genetics and Secondary Cell-Wall Polymers

- At the N-terminal part, there are **three repeats of S-layer homology (SLH) motifs**, consisting of 50–60 amino acids each
- **SLH motifs involved in Secondary Cell-Wall Polymers(SCWP)-mediated anchoring of the S-layer protein to the peptidoglycan layer**
- A highly **specific lectin type recognition mechanism** between the S-layer protein and a distinct type of SCWP.
- The interaction of the S-layer protein SbsB of *G. stearothermophilus* PV72/p2 and SCWP was assessed by surface plasmon resonance (SPR) biosensor technology.
- By using two truncated forms consisting either of the three SLH motifs or the residual part of SbsB, the complete responsibility of a functional domain formed by the three SLH motifs of the S-layer protein SbsB for SCWP recognition was clearly confirmed

Genetics and Secondary Cell-Wall Polymers

- The interaction was specific for the carbohydrate component, and strong evidence for glycan pyruvylation was provided.
- In contrast to most S-layer proteins of Gram-positive bacteria, those of *G. stearothermophilus* wild-type strains and *Lactobacillus* do not possess SLH-motifs.
- Nevertheless, the N-terminal part of *G. stearothermophilus* wild-type strains is conserved and recognizes a net negatively charged SCWP as binding site
- The production of different truncated forms of the S-layer protein SbsC of *G. stearothermophilus* ATCC 12980 confirmed that the N-terminal part is exclusively responsible for cell-wall binding.
- This positively charged N-terminal segment not involved in self-assembly and folds independently of the remainder protein sequence.

Structure-function Relationships of S-layer

- To determine at which amino acid positions of the S-layer proteins foreign peptide sequences could be fused without interfering with the self-assembly and recrystallization properties, the structure–function relationship of distinct segments of different S-layer proteins had to be elucidated.
- In S-layer protein SbpA of *Bacillus sphaericus* CCM 2177, **the C-terminal end of full-length form of recombinant rSbpA (rSbpA₃₁₋₁₂₆₈) was only available to a limited extent, but was fully accessible in the C-terminally truncated form rSbpA₃₁₋₁₀₆₈**
- **C-terminally truncated form a base for construction of S-layer fusions proteins, incorporating either the major birch pollen allergen Bet v1 (rSbpA31-1068 /Bet v1) or a camel antibody sequence recognizing lysozyme as an epitope(rSbpA31-1068 /cAB)**
- Using streptavidin–biotin interaction as a biomolecular coupling system, **minimum-sized core-streptavidin (118 amino acids) was fused either to N-terminal positions of the S-layer protein SbsB or attached to the C-terminus of this S-layer protein**

Chimeric S-layer

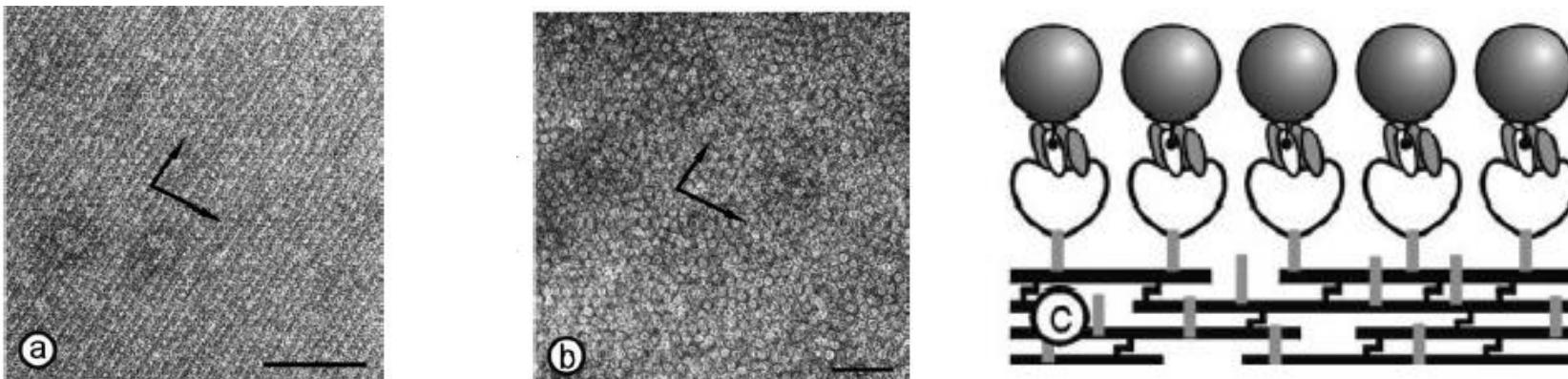


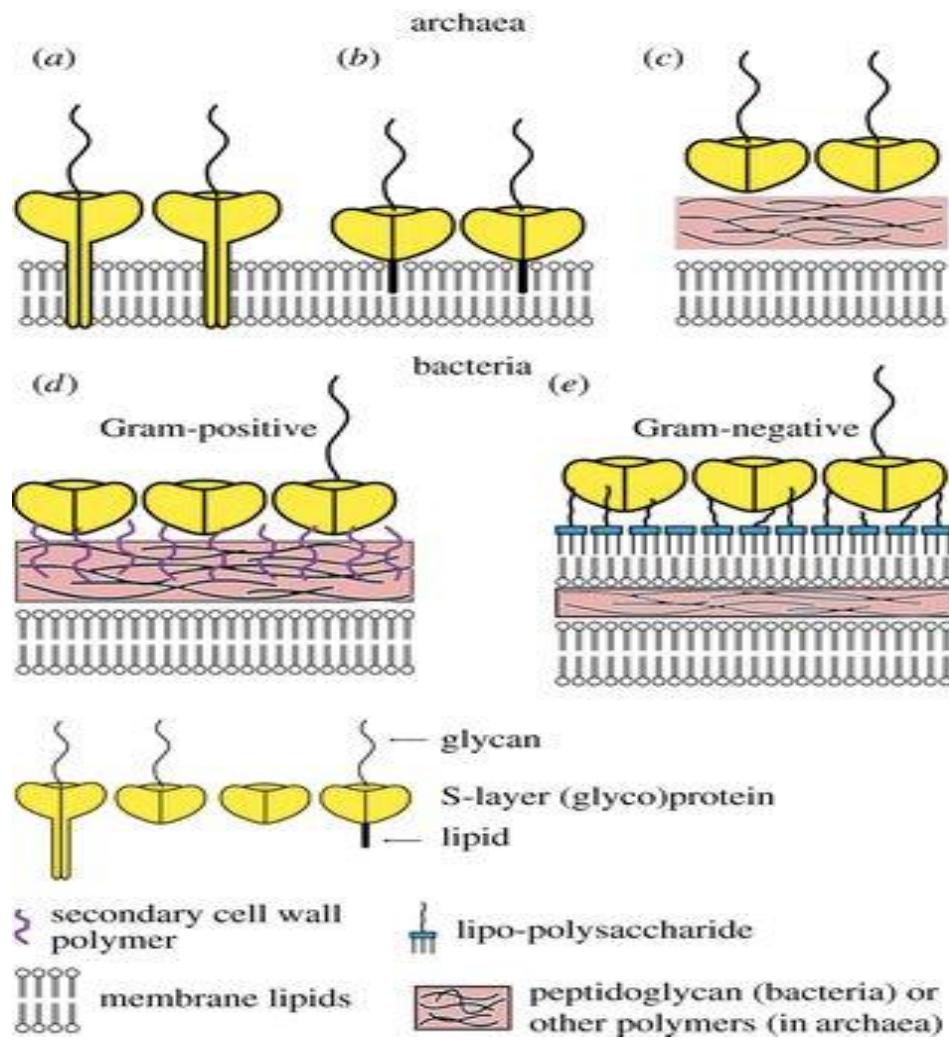
Figure : Cell wall fragments carrying a **chimeric S-layer formed by the fusion protein BS1(S1)₃** (a) were capable of binding **biotinylated ferritin** (b). At BS1(S1)3, one core streptavidin is fused to the C-terminus of the S-layer protein SbsB of *Geobacillus stearothermophilus* PV72/p2. The proteins were refolded to heterotetramers consisting of one chain of fusion protein and three chains of streptavidin. (a) Self-assembly was enabled by the specific interaction between an accessory cell-wall polymer that is part of the cell wall of *G. stearothermophilus* PV72/p2, and the SLH domain of the fusion protein. (b) Bound biotinylated ferritin reflected the underlying S-layer lattice. The preparations were negatively stained with uranyl acetate for TEM. The arrows indicate the base vectors of the oblique p1 lattice; scale bars =100 nm. (c) The cartoon shows the orientation of BS1(S1)₃ after SLH-enabled self-assembly with the streptavidin carrying outer face of the S-layer exposed.(Reproduced from Ref. [45]; copyright (2002) National Academy of Sciences,USA.)

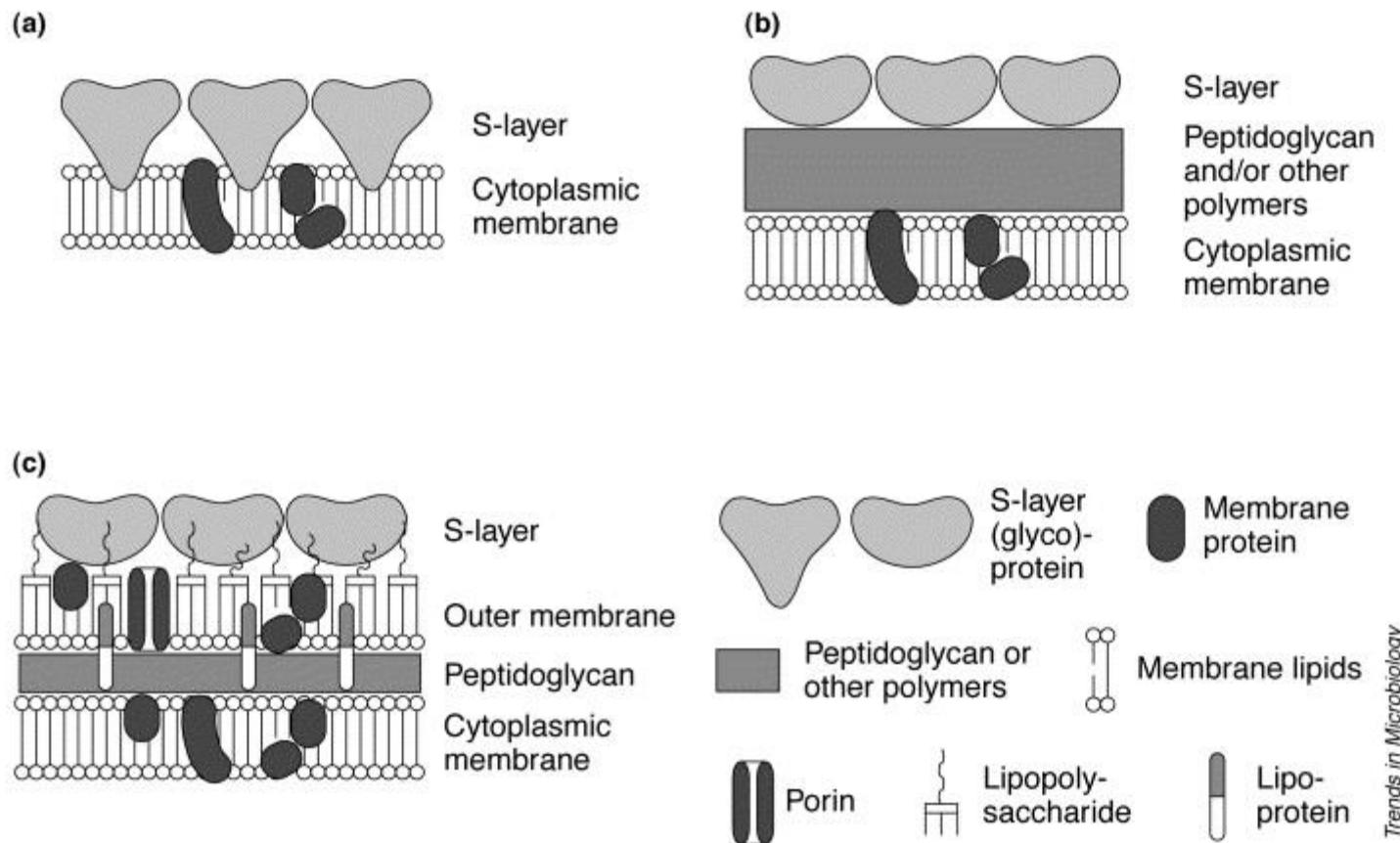
Features of fusion protein

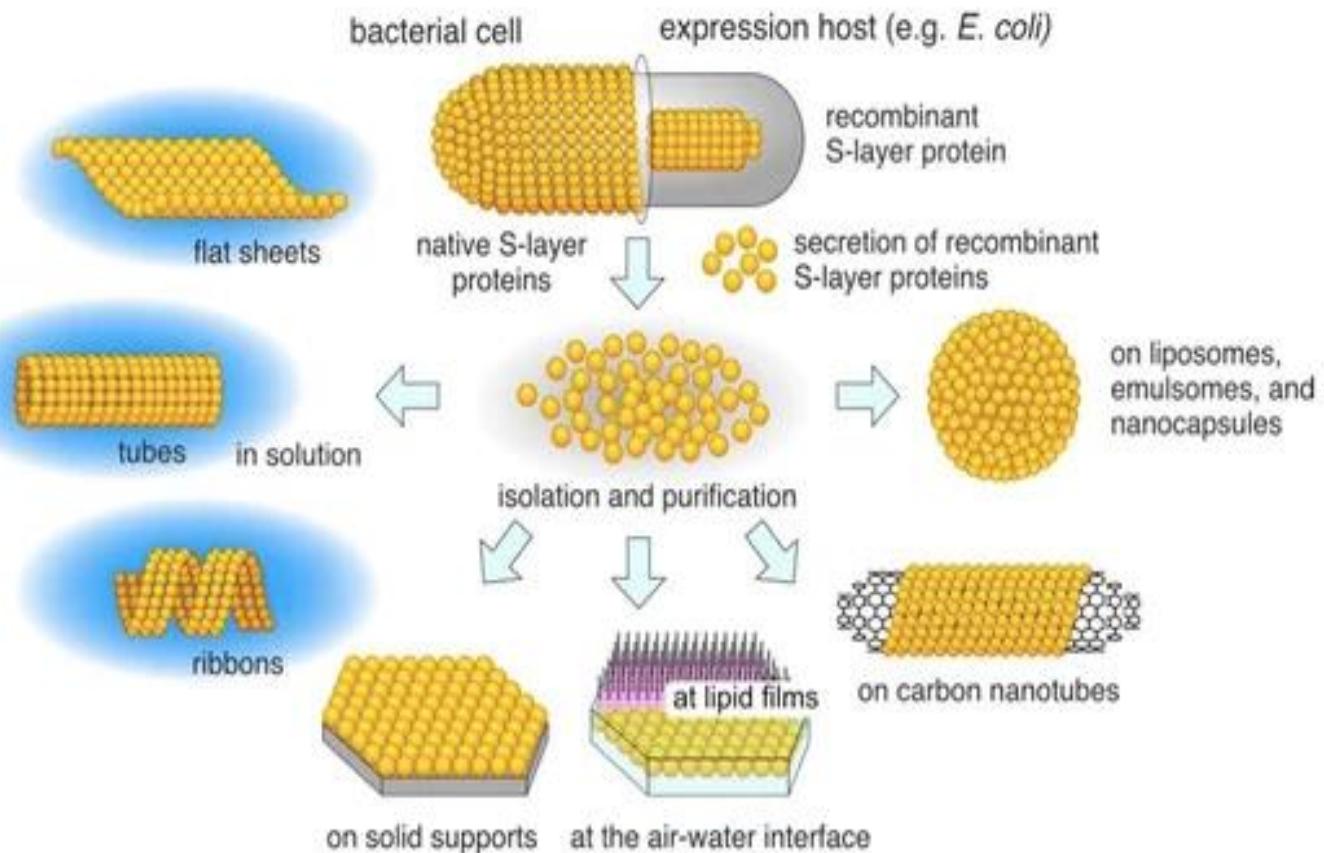
- The fusion proteins and core-streptavidin were produced independently in *Escherichia coli*, isolated and refolded to heterotetramers consisting of one chain of fusion protein and three chains of streptavidin.
- As determined by a fluorescence titration method, the biotin binding capacity of the heterotetramers was 80% in comparison to homotetrameric streptavidin, indicating that at least three of the four core streptavidin residues were accessible and active.
- Due to the ability of the heterotetramers to recrystallize in suspension, on liposomes, and on silicon wafers, this chimeric S-layer can be used as self-assembling nanopatterned molecular affinity matrix to arrange biotinylated compounds on a surface

Assembly

- A complete solubilization of S-layers into their constituent subunits achieved with high concentrations of hydrogen bond-breaking agents (e. g., guanidine hydrochloride).
- From results of disintegration procedures, it was concluded:
 - S-layer proteins not covalently linked to each other or supporting cell wall
 - Combinations of weak bonds (hydrophobic bonds, ionic bonds, and hydrogen bonds), responsible for the structural integrity of S-layers
 - Bonds holding the S-layer subunits together are stronger than those binding the S-layer lattices to the underlying envelope layer or membrane

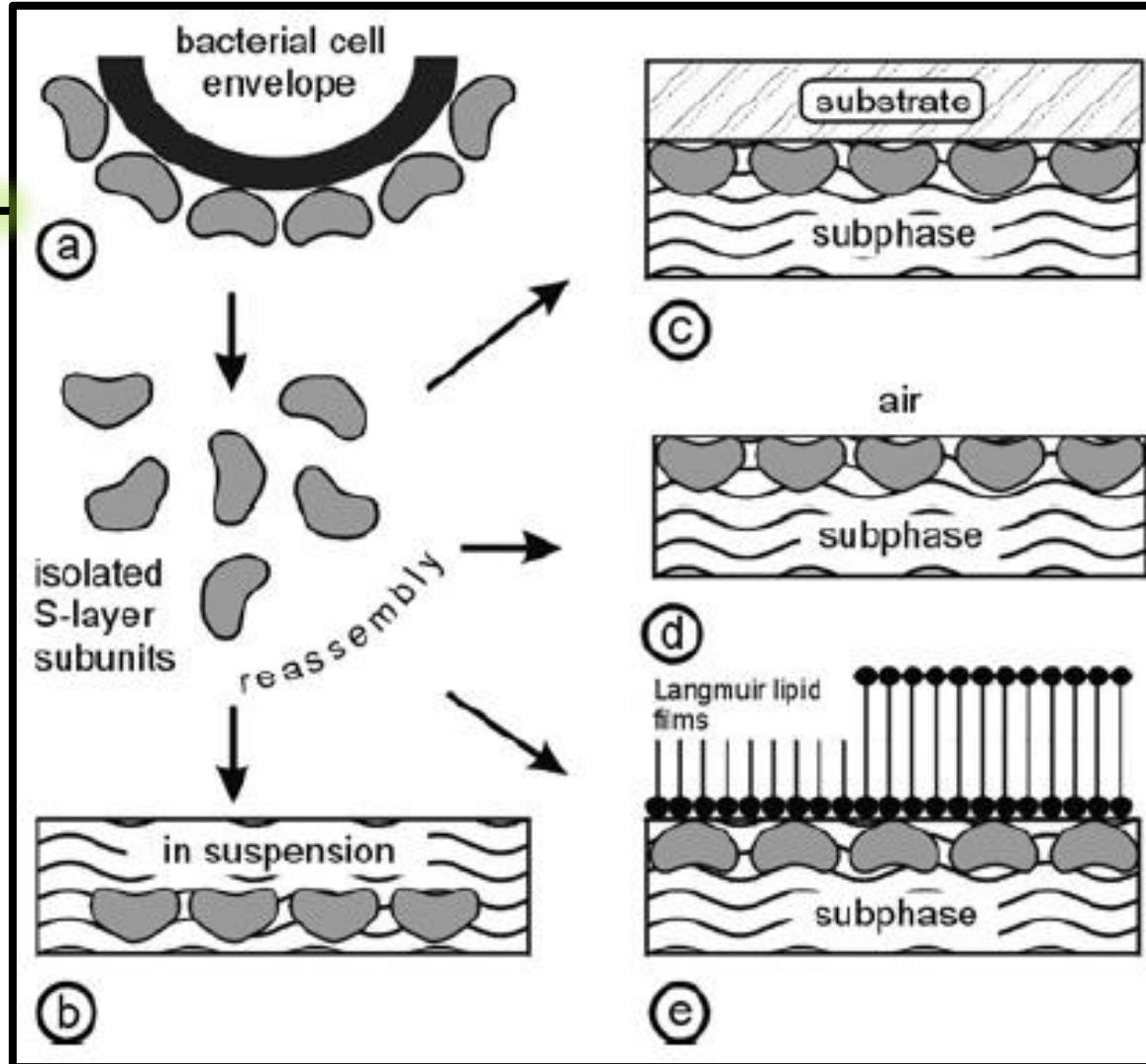






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Figure (a) Schematic illustration of the recrystallization of isolated S-layer subunits into crystalline arrays. The self assembly process can occur in suspension (b), on solid supports (c), at the air/water interface (d), and on Langmuir lipid films (e). (Reproduced from Ref. [3], with permission from Wiley-VCH.)



Self-Assembly in Suspension

- S-layers have ability to reassemble into two-dimensional arrays after removal of the disrupting agent
- High-resolution electron microscopical studies showed, crystal growth is initiated simultaneously at many randomly distributed nucleation points and proceeds in-plane until the crystalline domains meet, thus leading to a closed, coherent mosaic of individual several micrometer large S-layer domains
- **Formation of these self-assembled arrays is only determined by the amino-acid sequence of the polypeptide chains and, consequently, the tertiary structure of the S-layer protein species.**
- The self-assembly products may have the form of **flat sheets, open-ended cylinders or closed vesicles.**
- The **shape and size** of the self-assembly products depends strongly on the **environmental parameters during crystallization such as temperature, pH, ion composition, and/or ionic strength.**

Recrystallization at Solid Supports

- Reassembly of isolated S-layer proteins can be induced on solid surfaces
- Recrystallization of S-layer proteins on technologically relevant substrates such as silicon wafers, carbon-, platinum- or gold electrodes and on synthetic polymers revealed a broad application potential in micro- and nanotechnology
- Formation of coherent crystalline arrays depends on
 - S-layer protein species
 - Environmental conditions of the bulk phase
 - Surface properties of the substrate

Recrystallization at Solid Supports

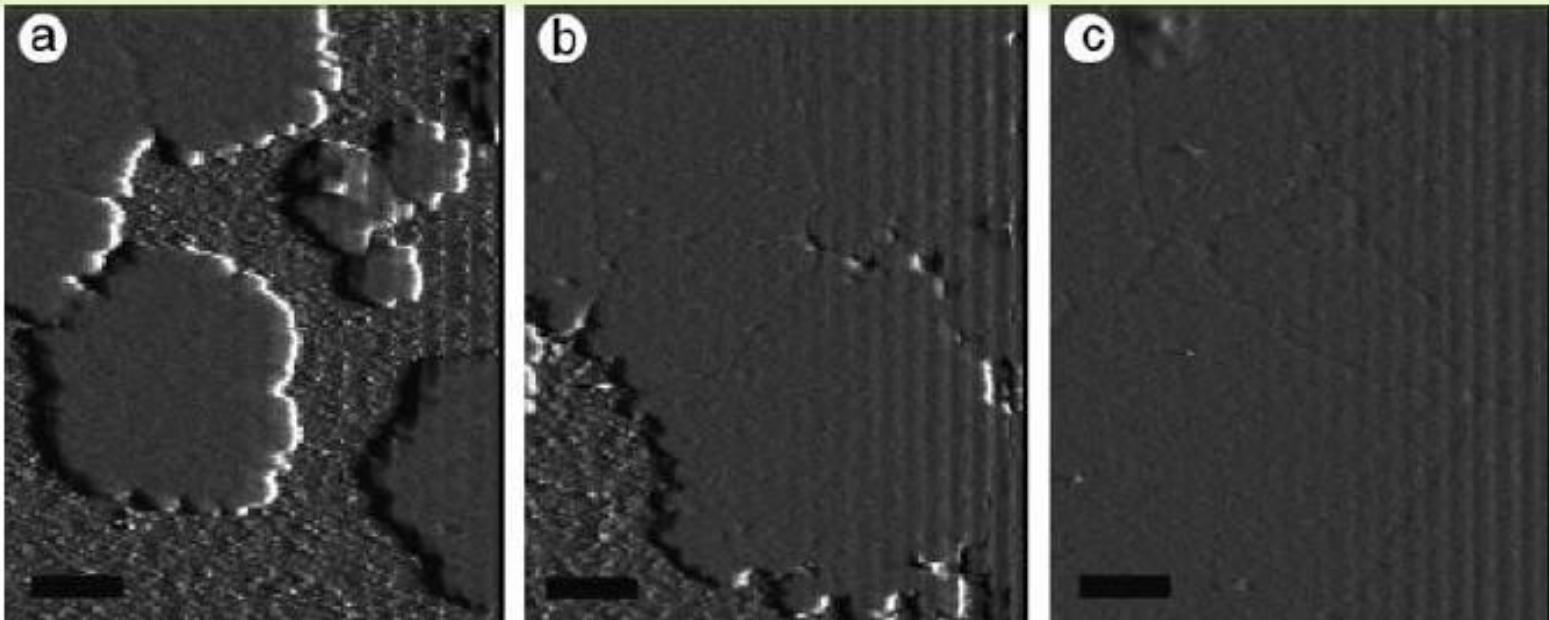


Figure: Recrystallization of the S-layer protein SbpA of *Bacillus sphaericus* CCM 2177 on a hydrophilic silicon wafer. The atomic force microscopical images show that crystal growth is initiated simultaneously at many randomly distributed nucleation points (a) and proceeds in-plane until the crystalline domains meet (b), thus leading to a closed, coherent mosaic of individual several micrometer large S-layer domains (c). Scale bars = 0.5 mm; Z-range = 12 nm. (Figure courtesy of E. Györvary and O. Stein.)

- Langmuir-Blodgett (LB) films are prepared by transferring floating organic monolayers onto solid substrates.
- A combination of innovative chemistry and a carefully engineered instrument (a Langmuir trough) can result in high quality monomolecular assemblies displaying a high degree of structural order.
- The technique was first reported about fifty years ago and since then, LB films have been widely used as model systems in fundamental research.
- However, it is only during the past decade that the extensive applied potential has been recognised.
- Their precise thickness, coupled with the degree of control over the molecular architecture, has now firmly established a role for these layers in thin film technology.
- Potential applications in molecular electronics

Recrystallization at the Air/Water Interface and on Langmuir Lipid

Films

- It is easy and reproducible way to generate coherent S-layer lattices on a large scale.
- The orientation of the protein arrays at liquid interfaces is determined by the anisotropy in the physico-chemical surface properties of the protein lattice.
- Electron microscopical examinations revealed, recrystallized S-layers were oriented with
 - Their outer charge neutral, more hydrophobic face against the air/water interface
 - Negatively charged, more hydrophilic inner face against charge neutral, charged or zwitterionic headgroups of phospho- or tetraether lipid films
- As with S-layer lattices recrystallized on solid surfaces, S-layer protein monolayers consist of a closed mosaic of individual monocrystalline domains.

Diagnostics

- Studies on the structure, morphogenesis, genetics, and function of S-layers revealed that
- S-Layer iso-porous monomolecular arrays have a considerable **application potential in biotechnology, molecular nanotechnology, and biomimetics.**
- The repetitive features of S-layers have led to their applications in **S-layer ultrafiltration membranes (SUMs)**
- Supports for a defined **covalent attachment of functional molecules e.g.**
- Enzymes, antibodies, antigens, protein A, biotin, and avidin
- Required for affinity and enzyme membranes, in the development of **solid-phase immunoassays, or in biosensors**

SUM-based dipsticks

- In dipstick-style solid-phase immunoassays, the respective monoclonal antibody was covalently bound to the carbodiimide-activated carboxylic acid groups of the S-layer lattice
- Proof of principle demonstrated for different types of SUM-based dipsticks.
 - **Diagnosis of type I allergies** (determination of IgE in whole blood or serum against the major birch pollen allergen Bet v1),
 - **Quantification of tissue type plasminogen activator (t-PA)** in patients' whole blood or plasma
 - **Monitoring t-PA** levels during the course of thrombolytic therapies after myocardial infarction
 - **Determination of interleukin 8 (IL-8)** in the supernatants of human umbilical vein endothelial cells induced with lipopolysaccharides

Chimeric S-layer fusion proteins

- Chimeric S-layer fusion proteins are genetically constructed by incorporating biologically active sequences without hindering the self-assembly of S-layer subunits
- Chimeric S-layer proteins rSbsC₃₁₋₉₂₀/Bet v1 and rSbpA₃₁₋₁₀₆₈/Bet v1 carrying the major birch pollen allergen Bet v1 at the C-terminal end, the surface location and functionality of the fused allergen was demonstrated by binding Bet v1-specific IgE.
- These fusion proteins can be used for building up arrays for diagnostic test systems to determine the concentration of Bet v1-specific IgE in patients' whole blood, plasma, or serum samples.
- To build up functional monomolecular S-layer protein lattices on solid supports (e.g., gold, silicon, or glass), the surface must be functionalized with covalently attached chemically modified SCWP, to which the S-layer fusion proteins bind with their N-terminal part, leaving the C-terminal part with the fused functional sequence exposed to the ambient environment.

Label-free detection systems

- Using **streptavidin–biotin interaction** as a biomolecular coupling system, S-layer-streptavidin fusion proteins were constructed
- The chimeric S-layer, a feasible tool **to arrange different biotinylated targets** (e. g., proteins, allergens, antibodies, or oligonucleotides) on a surface which will find application in protein, allergy, or DNA-chip technology, highly integrated diagnostic devices (Lab-on-Chip).
- Another application is in the development of **label-free detection systems**,
 - In SPR or surface acoustic wave technique, specific binding of functional molecules (e. g., proteins or antibodies) to the sensor chip functionalized with an oriented chimeric S-layer can be visualized directly by a mass increase on the chip without the need for any labeled compound.
- Thus, a functional S-layer fusion protein recrystallized in defined orientation on SCWP-coated solid supports allow the development of new label-free detection systems as **required for biochip technology**.

Lipid Chips: Stabilization of bilayer lipid membranes (BLMs)

- Free-standing bilayer lipid membranes (BLMs) survive for only minutes to hours and sensitive toward vibration and mechanical shocks
- Stabilization of BLMs is necessary to utilize the function of cell membrane components for practical applications (e. g., as lipid chips).
- S-layer proteins can be exploited as supporting structures for BLMs since they stabilize lipid film and retain their physical features (e. g., thickness, fluidity)
- Lipid membranes attached to a porous support combine the advantage of possessing an essentially unlimited ionic reservoir on each side of the lipid membrane and of easy manual handling

SUM-supported bilayers

- A new strategy is **application of SUM with the S-layer as stabilizing and biochemical layer between the BLM and the porous support.**
- SUMs are iso-porous structures with sharp molecular exclusion limits
- SUM are manufactured by depositing S-layer-carrying cell wall fragments under high pressure on commercial microfiltration membranes (MFM)s with pore size of ~ 0.4 mm
- After deposition, the S-layer lattices are chemically crosslinked to form a coherent smooth surface ideal for depositing lipid membranes.
- **Composite SUM-supported bilayers (Figure) are tight structures with breakdown voltages well above 500 mV during their whole life-time of 8 ~ hours**
- For a comparison, lipid membranes on a plain nylon MFM have a life-time of 3 hours, ruptured at breakdown voltages ~ 210 mV.

SUM-supported bilayers

- Specific capacitance measurements and reconstitution experiments revealed functional lipid membranes on the SUM as the **pore-forming protein α -hemolysin could be reconstituted to form lytic channels.**
- For the first time, the opening and closing behavior of even single α -hemolysin pores could be measured with membranes generated on a porous support
- The main phospholipid of *Thermoplasma acidophilum* (MPL), membrane-spanning tetraether lipid, transferred on an SUM using a modified Langmuir–Blodgett technique
- SUM-supported MPL-membranes allowed reconstitution of functional molecules, as proven by measurements on single gramicidin pores.
- Recrystallization of an additional monomolecular S-layer protein lattice on the lipid-faced side of SUM-supported MPL membranes increased the lifetime to 21.2 ± 3.1 hours

Solid-supported lipid membranes

- Solid-supported membranes (Figure D) were developed to
 - **overcome fragility** of free-standing BLMs
 - to enable biofunctionalization of inorganic solids (e. g., semiconductors, gold-covered surfaces) for **use in sensing devices** e.g., lipid chips.
- Solid-supported lipid membranes often show drawbacks
 - there is a **limited ionic reservoir** at the side facing the solid support
 - the membranes often appear to be **leaky (non-insulating)**,
 - large domains, protruding from the membrane, may become denatured by the inorganic support
- Here also, **S-layer proteins** have potential as **stabilizing, separating ultrathin layer and maintains structural and dynamic properties of the lipid membranes**.
- Silicon substrates covered by closed S-layer lattice and bilayers were deposited by Langmuir–Blodgett technique

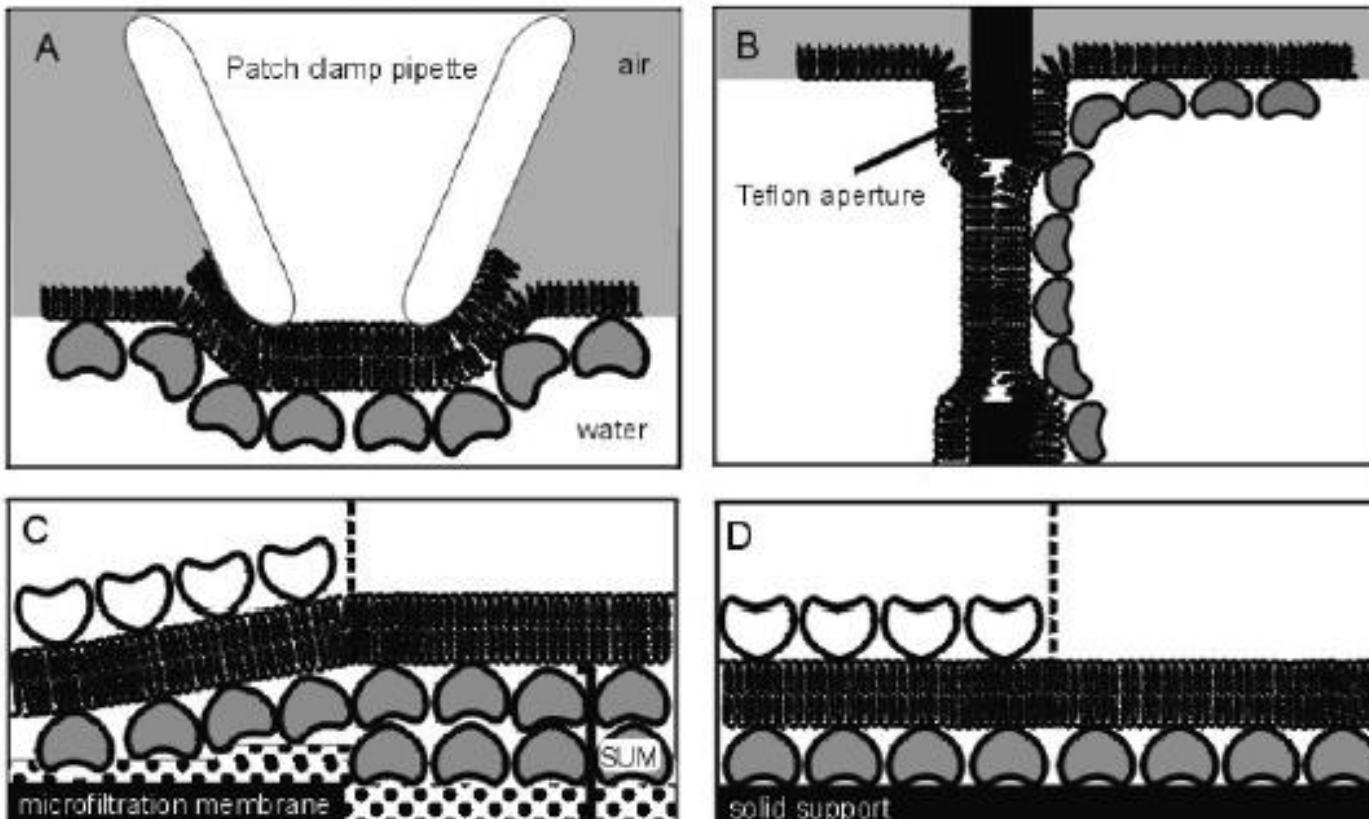


Figure: Schematic illustrations of various S layer- supported lipid membranes. (A) Bilayer lipid membranes (BLMs) have been generated across an aperture of a patch-clamp pipette using the Tip-Dip method, and a closed S-layer has been recrystallized from the aqueous subphase. (B) A folded membrane has been generated to span a Teflon aperture using the method of Montal and Mueller. Subsequently, S-layer protein can be injected into one or both compartments (not shown), whereby the protein self-assembles to form closely attached S-layer lattices on the BLMs. (C) On an S-layer ultrafiltration membrane (SUM) a BLM can be generated by a modified Langmuir-Blodgett (LB) technique. As a further option, a closed S-layer lattice can be attached on the external side of the SUM-supported BLM (left part). (D) Solid supports can be covered by a closed S-layer lattice, and subsequently BLMs can be generated using combinations of the LB and Langmuir-Schaefer techniques, and vesicle fusion. As shown in (C), a closed S-layer lattice can be recrystallized on the external side of the solid supported BLM (left part).

S layer- supported lipid membranes

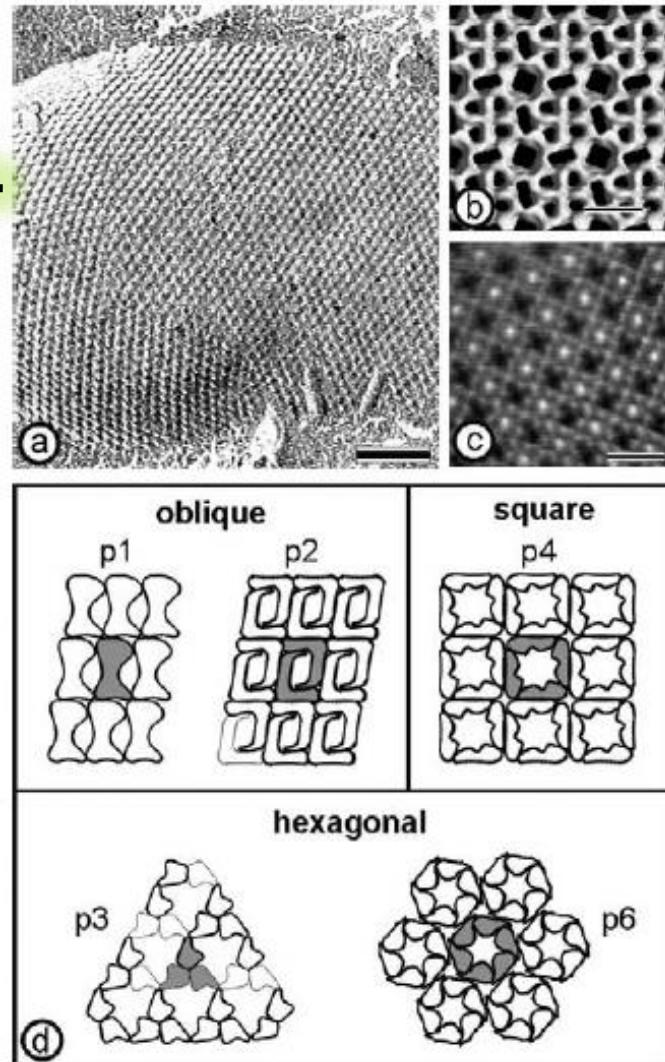
- Lateral diffusion of fluorescently labeled lipid molecules in both layers, investigated by fluorescence recovery after photobleaching studies
 - In comparison with hybrid lipid bilayers (lipid monolayer on alkyl silanes) and lipid bilayers on dextran, **the mobility of lipids was highest in S-layer-supported bilayers.**
- S-layer cover **prevent the formation of cracks** and inhomogeneities in the bilayer
- Concluding, the biomimetic approach of copying the supramolecular architecture of archaeal cell envelopes opens new possibilities for exploiting functional lipid membranes at meso- and macroscopic scale.
- It has potential to initiate a broad spectrum of lipid chips applicable for sensor technology, diagnostics, electronic or optical devices, and high-throughput screening for drug discovery.

S-Layers as Templates for the Formation of Regularly Arranged Nanoparticles

- The **reproducible formation of nanoparticle arrays in large scale with predefined lattice spacing and symmetries remains a challenge** in the development of future generations of molecular electronic devices
- Biomolecular templating has proven to be very attractive, as the **self-assembly of molecules into monomolecular arrays is an intrinsic property of many biological molecules** and has already grown into a scientific and engineering discipline.
- The first approach in using S-layers as templates in the generation of perfectly ordered nanoparticle arrays was developed by Douglas and coworkers
 - S-layer fragments of *Sulfolobus acidocaldarius* were deposited on a smooth carbon surface and metal coated by evaporation of a ~ 1 nm-thick tantalum/tungsten film.
 - Then, this protein–metal heterostructure was ion milled, leading to 15 nm-sized holes hexagonally arranged according to the center-to-center spacing of the S-layer of 22 nm.

Structure

Figure: (a) Freeze-etching preparation of whole cells of *Thermoanaerobacter thermohydrosulfuricus* L111-69 revealing a hexagonally ordered array. Scale bar = 100nm. (b) Three-dimensional model of the S-layer of *Bacillus stearothermophilus* NRS 2004/3a/V2 exhibiting oblique lattice symmetry. The protein meshwork shows one square shaped, two elongated, and four small pores per morphological unit. (c) Computer image reconstruction of scanning force microscopic images of the topography of the square S-layer lattice from *Bacillus sphaericus* CCM 2177. The images were taken under water. The surface corrugation corresponding to a gray scale tram black to white is 1.8 nm. Scale bars in (b) and (c) = 10 nm. (d) Schematic drawing of the different S-layer lattice types. The regular arrays exhibit either oblique (p1, p2), square (p4), or hexagonal lattice symmetry (p3, p6). The morphological units are composed of one, two, three, four, or six identical subunits. (Reproduced from Ref. [3], with permission from Wiley-VCH.)



S-Layers as Templates for the Formation of Regularly Arranged Nanoparticles

-
- The approach was further optimized using S-layer on a smooth graphite surface and titanium oxide for the metal coating
 - After oxidation in air and fast-atom beam milling at normal incidence, a thin ~ 3.5 nm metallic nano-porous mask with pores of 10 nm.
 - The same group used low-energy electron-enhanced etching to pattern the surface properties of a silicon substrate through the regularly arranged pores of the S-layer
 - After etching and removal of the S-layer, the patterned surface was oxidized in an oxygen plasma, leading to a nanometric array of etched holes (18 nm diameter) which served as nucleation sites in the formation of an ordered array of nanometric titanium metal clusters.
 - In a similar approach using argon ion etching, the S-layer of *Deinococcus radiodurans* was used as a nanometric template for patterning ferromagnetic films
 - Uniform hexagonal patterns of 10 nm-wide dots and lattice spacing of 18 nm were fabricated from 2.5 nm-thick sputter-coated Co, FeCo, Fe, FeNi, and NiFe films.

S-Layers as Templates for the Formation of Regularly Arranged Nanoparticles

- A synthesis pathway for fabrication of nanoparticles by wet chemical processes and S-layers as nanometric templates was developed
- Self-assembled S-layer were exposed to a metal–salt solution (e. g., $[\text{AuCl}_4]^-$, $[\text{PtCl}_4]_2^-$), followed by slow reaction with reducing agent such as hydrogen sulfide (H_2S).
- Nanoparticle superlattices were formed according to the lattice spacing and symmetry of the underlying S-layer.
- Since the precipitation of the metals was confined to pores of S-layer, nanoparticles also resembled morphology of pores
- The first example exploiting this technique was the precipitation of cadmium sulfide (CdS) on S-layer lattices composed of SbsB and SbpA
 - After incubation of the S-layer self-assembly products with a CdCl_2 solution for several hours, the hydrated samples were exposed towards H_2S for one or two days.
 - The generated CdS nanoparticles were 4–5 nm in size, and their superlattice resembled the oblique lattice symmetry of SbsB ($a = 9.4 \text{ nm}$, $b = 7.4 \text{ nm}$, $g = 80''$), or the square lattice symmetry of SbpA ($a = b = 13.1 \text{ nm}$, $g = 90''$), respectively.

S-Layers as Templates for the Formation of Regularly Arranged Nanoparticles

- In a similar approach, a **superlattice of 4–5 nm-sized gold particles** was formed by using SbpA (with previously induced thiol groups) as a template for precipitation of a tetrachloroauric (III) acid solution(Figure).
- Gold nanoparticles formed either by reduction of the metal salt with H_2S or under the electron beam in a transmission electron microscope.
- The latter approach is technologically important as it allows those areas where nanoparticles are formed to be defined.
- As determined by electron diffraction, the gold nanoparticles were crystalline but their ensemble was not crystallographically aligned.
- The wet chemical approach was used in the formation of Pd- (salt: PdCl_2), Ni- (NiSO_4), Pt- (KPtCl_6), Pb- ($\text{Pb}(\text{NO}_3)_2$) and Fe- ($\text{KFe}(\text{CN})_6$) nanoparticle arrays, and for producing platinum nanoparticles on the S-layer of *Sporosarcina ureae*

S-Layers as Templates for the Formation of Regularly Arranged Nanoparticles

- Unfortunately, **wet chemical methods do not allow varying size or composition of nanoparticles in the fabrication process**. Thus, the **binding of preformed nanoparticles into regular arrays on S-layers has significant advantages** in the development of nanoscale electronic devices
- Similar to binding biomolecules (e. g. enzymes or antibodies) onto S-layers, gold or CdSe nanoparticles can be electrostatically bound in regular arrangements on S-layers
- The nanoparticles were either negatively charged due to surface citrate ions or positively charged due to surface coating with poly-L-lysine
- In summary, these experiments have clearly shown that S-layers are suited to control the formation of nanoparticle arrays, either by direct precipitation from the vapor or liquid phase, or by binding preformed nanoparticles
- The S-layer approach provides for the first time a biologically based fabrication technology for self-assembly of molecular electronic or optic devices

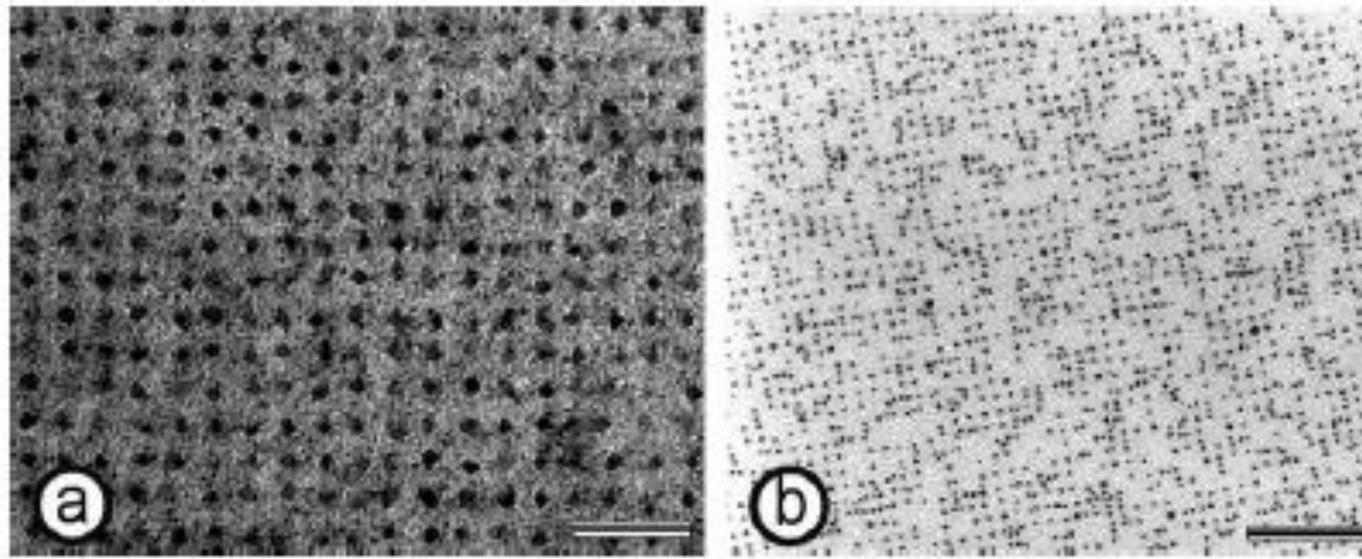


Figure (a) Electron microscopical image of **gold nanoparticles (mean diameter 4.5 nm) obtained using wet chemistry**. An S-layer with square lattice symmetry served as template in the precipitation of the metal salt. The gold nanoparticles were formed in the pore region of the protein meshwork under the electron beam. Scale bar = 50 nm.

(b) Electron microscopical image of **preformed gold nanoparticles (mean diameter 4 nm) regularly bound on the surface of an S-layer with square lattice symmetry**. Electrostatic interactions between the surface of the nanoparticles and functional domains on the S-layer are responsible for the binding. Scale bar = 100 nm.
(Reproduced from Ref. [91], with permission from Elsevier.)

Conclusion

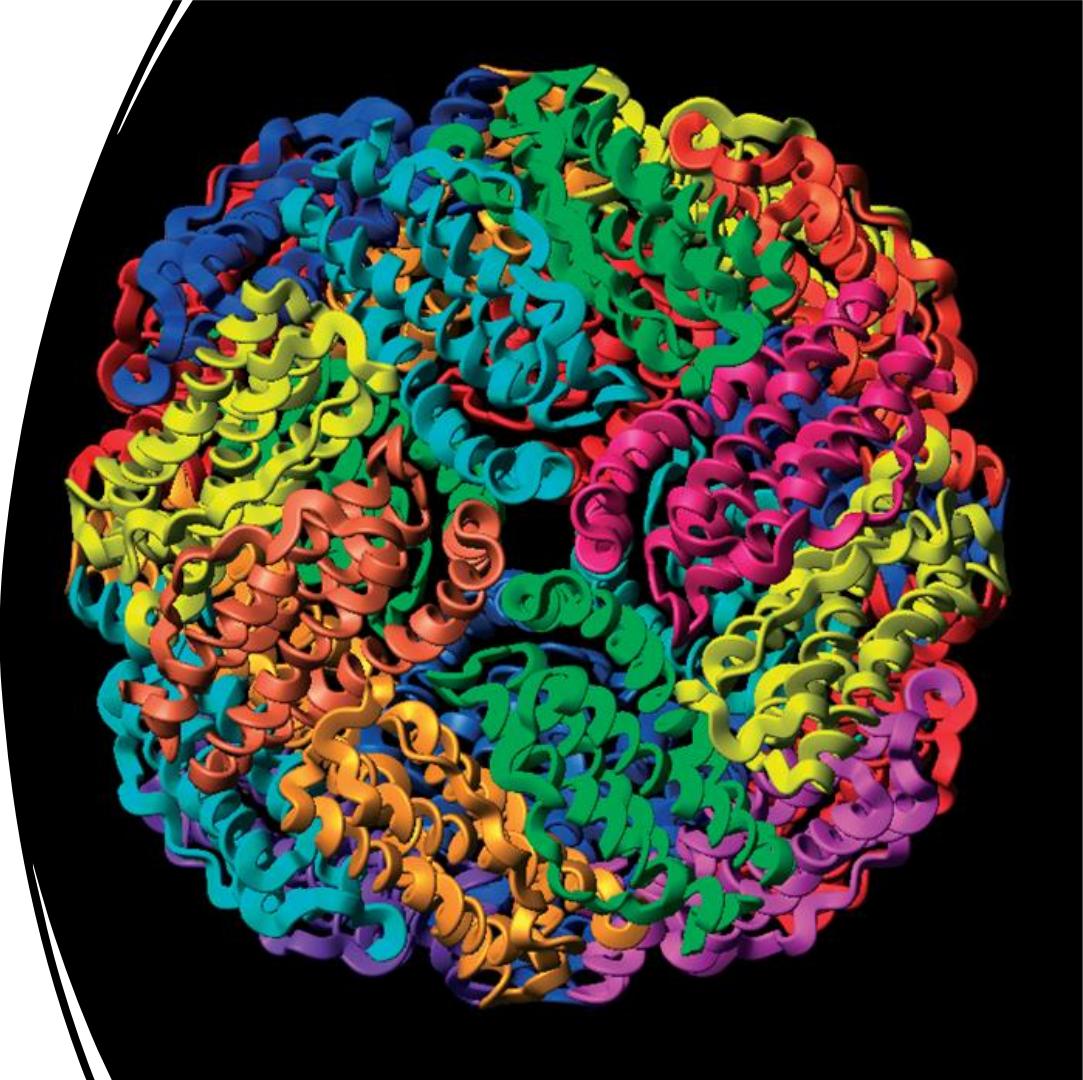
- At present, most applications developed for using S-layers depend on the in vitro self-assembly capabilities of native S-layer proteins in suspension, on the surface of solids (e.g., silicon wafers, metals, polymers), Langmuir-lipid films, and liposomes.
- Once the regular arrays have been formed, a broad spectrum of very precise chemical modifications can be applied for tailoring the physico-chemical properties of S-layers and for a defined binding of differently sized functional molecules.
- In particular, the possibility of immobilizing or growing other materials (e. g., silicon oxide, metals) on top of recrystallized S-layer lattices with most accurate spatial controlled architecture opens up many new possibilities in **nanofabrication** and **supramolecular engineering**
- An important line of development for the specific tuning of structural and functional features concerns the genetic manipulation of S-layer proteins.

Conclusion

- Recent studies have clearly demonstrated that **truncated S-layer proteins incorporating specific functional domains of other proteins maintain the self-assembly capability into regular arrays**
- This **approach can lead to new iso-porous ultrafiltration membranes**, affinity structures, enzyme membranes, ion- and metal particle-selective binding matrices, microcarriers, biosensors, diagnostics, biocompatible surfaces, and vaccines
- Moreover, biomimetic approaches copying the supramolecular principle of virus envelopes such as **S-layer-coated liposomes** will provide new strategies for **drug targeting and drug delivery**.
- Preliminary studies have also provided strong evidence that S-layers have a great potential as patterning elements for non-life science applications (e. g., nonlinear optics and molecular electronics)

Biomimetic Ferritins

Mineralization in Nanostructured
Bio compartments: Biomimetic
Ferritins For High-Density Data
Storage.



Ferritin

- Ferritin is a self-assembled, 12 nm-diameter multi-subunit protein involved in biological functions such as iron storage and heme production.
- 24 nearly identical subunits.
- Spherical shell that encloses an 8.0 nm-diameter internal cage.
- Cavity contains up to 4500 Fe atoms in the form of a poorly crystalline iron (III) oxy-hydroxide mineral, ferrihydrite.
- Apoferritin is robust, being able to withstand relatively high temperatures systems (65°C) and wide pH variations (approximately 4.0–9.0).

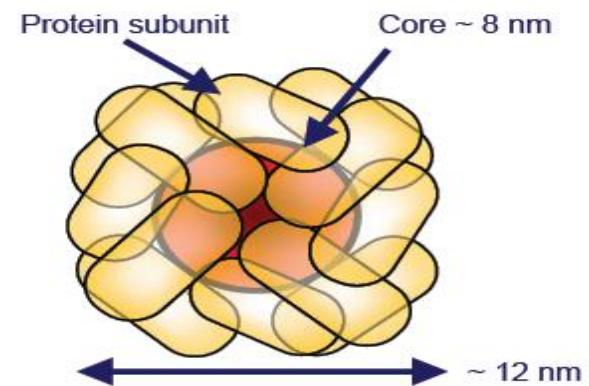
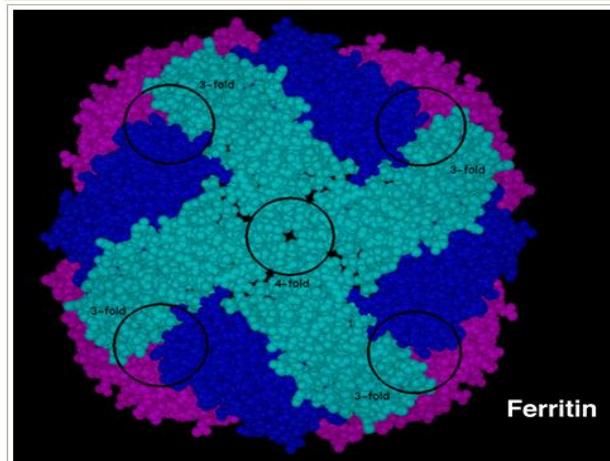


Figure 1. Schematic of the spherical shell of the ferritin protein.

Biomimetic ferritins

- Ferritin cage can act as generic reaction vessel.
- Biomimetic ferritins have been prepared by reductive dissolution of the iron oxide cores.
- Reconstitution of the empty cage of apoferritin with manganese oxide, uranyl oxide nanoparticles, cadmium sulfide quantum dots.
- Hybrid metals can also be employed like the synthesis of the magnetic alloy cobalt platinum (CoPt) within apoferritin which can help in high density data storage.

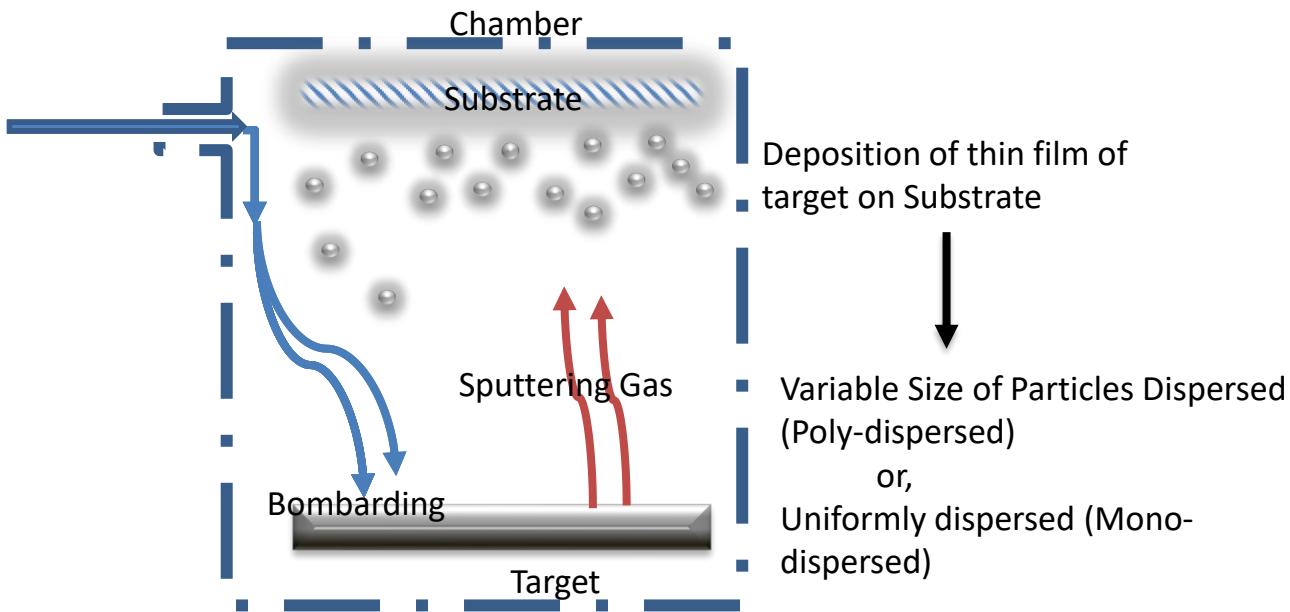
High-Density Magnetic Data

Storage

- Rectangular magnetized regions or ‘bits’ on a thin metal film supported by a glass or aluminum disk substrate.
- In general, storage capacity is about maximum areal density of around 11 Gbits cm^{-2} (i. e., billions of bits per cm^2)
- Bits are roughly 30 nm wide by 300 nm long
- Composed from hundreds of nano-scaled magnetic alloy grains oriented longitudinally in the plane of a disk.
- These grains are produced through a process of **sputtering**.

Sputtering

- **Ion Beam Sputtering**
- **Reactive Sputtering**
- **Gas Flow Sputtering**
- **High Power Impulse**



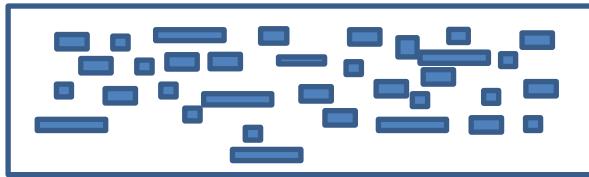
High-Density Magnetic Data

- Polydisperse grain size distribution.
- Storage (contd.)
- Critical to have a narrower grain size distribution when bit dimensions approach the average size of the grains (currently around 9 nm in diameter).
- The “**superparamagnetic limit**”
- Maximum density possible is anticipated to be in the region of 30 to 80 Gbits cm^{-2}

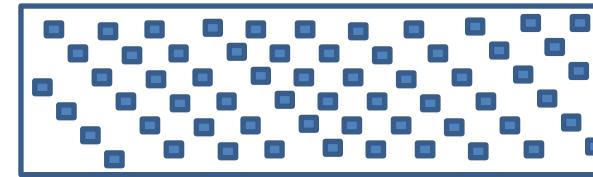
Superparamagnetic Limits:

- Most applications rely on the magnetic order of the nanoparticles being stable with time.
- However, with decreasing particle size, the magnetic anisotropy energy per particle responsible for holding the magnetic moment along certain directions becomes comparable to the thermal energy.
- When this happens, the thermal fluctuations induce random flipping of the magnetic moment with time, and the nanoparticles lose their stable magnetic order and become superparamagnetic.
- Thus, the demand for further miniaturization comes into conflict with the superparamagnetism caused by the reduction of the anisotropy energy per particle: this constitutes the so-called ‘superparamagnetic limit’ in recording media.

Polydispersity



Poly-dispersed



Mono-dispersed

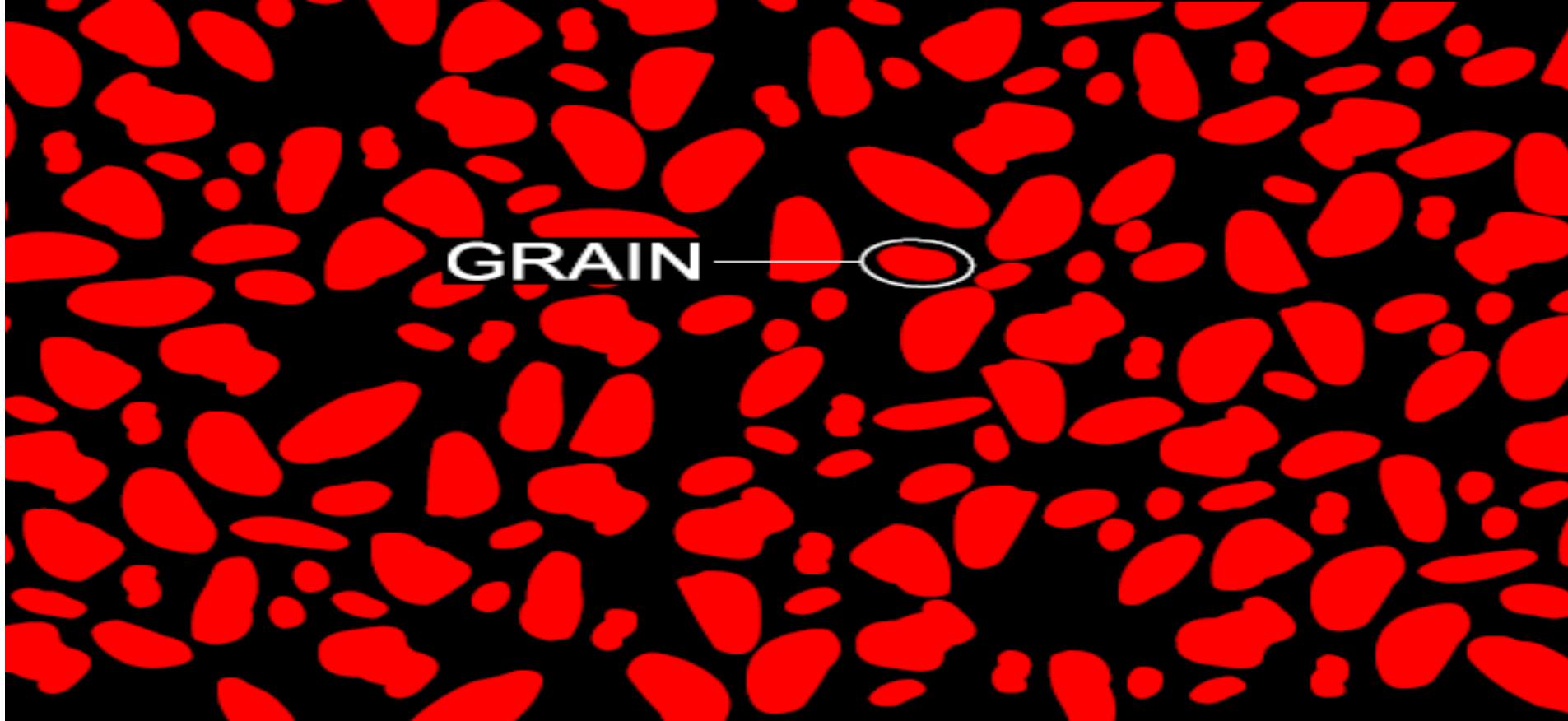
- “superparamagnetic limit” - 9nm

Ways to increase storage capacity

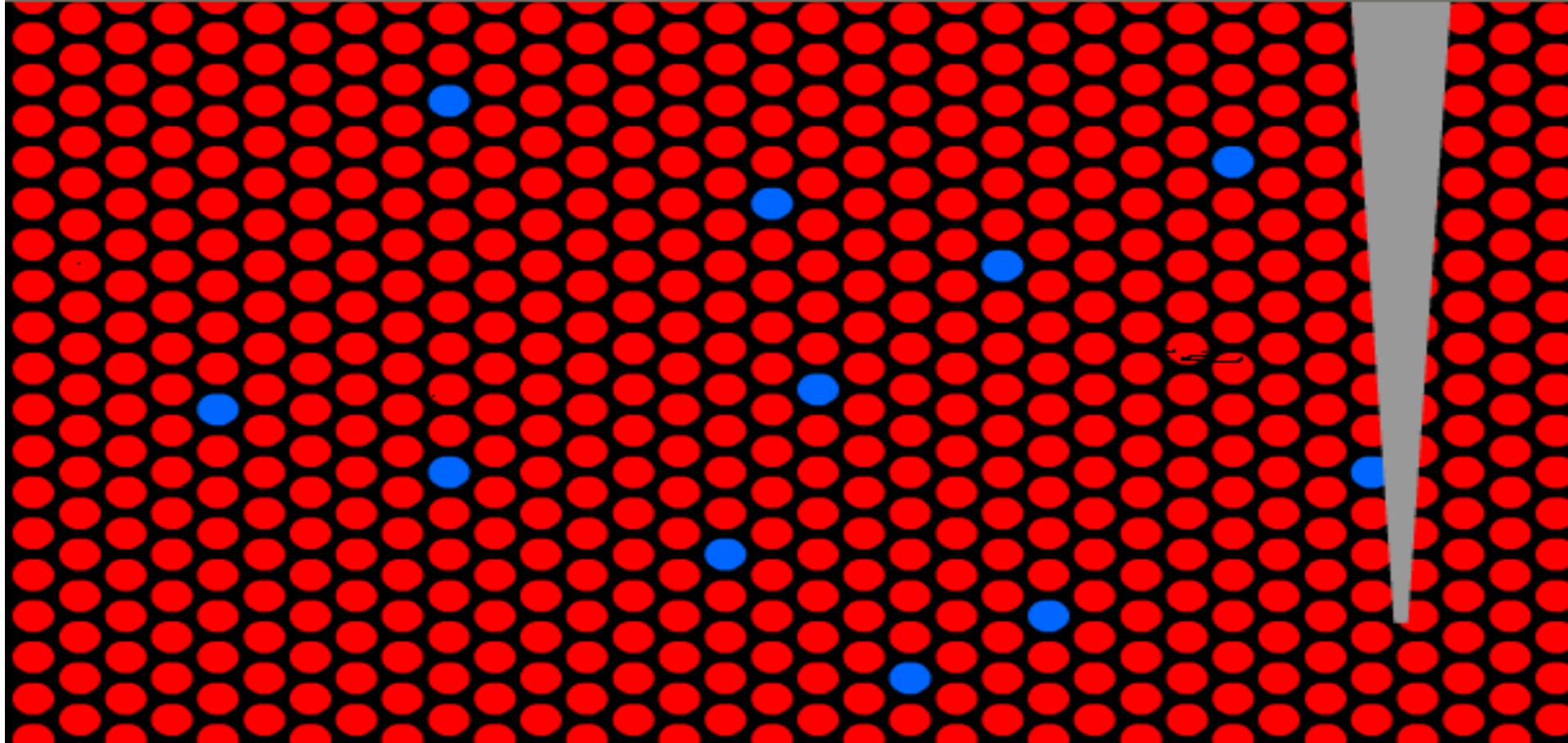
Reduce average size of magnetic grains

Make all grains uniformly small

Pattern the uniformly small grains in 3-D arrangements.



Irregular grain size leads to inefficient packing and low density data storage levels



Proposed high density biomimetic ferritin based data storage

High-Density Magnetic Data Storage (contd.)

- Sputtered thin films may have an average grain size larger than the superparamagnetic limit
- Inherent size distribution leaves a percentage which is thermally unstable.
- multilayer thin films that exploit antiferromagnetic coupling help to stabilize smaller grains.
- Also, longitudinal recording can be extended to 80 Gbits cm^{-2}

High-Density Magnetic Data Storage (contd.)

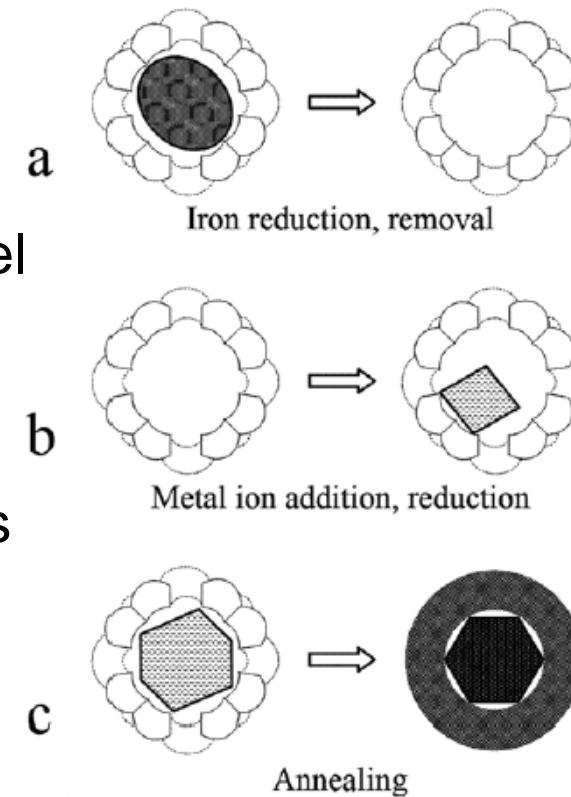
- Higher magnetocrystalline anisotropy are required for continually smaller, thermally stable grains.
- The most likely candidates to extend areal densities significantly beyond 30 Gbit cm^{-2} are thin films comprising monodisperse high anisotropy nanoparticles.
- Perpendicular magnetic orientation of grains have been proposed to extend areal densities up to 155 Gbit cm^{-2} – Polydispersed Grains.

Biomimetic Ferritins

Ferritin cage can act as a generic reaction vessel for nanoparticle synthesis

Steps involves

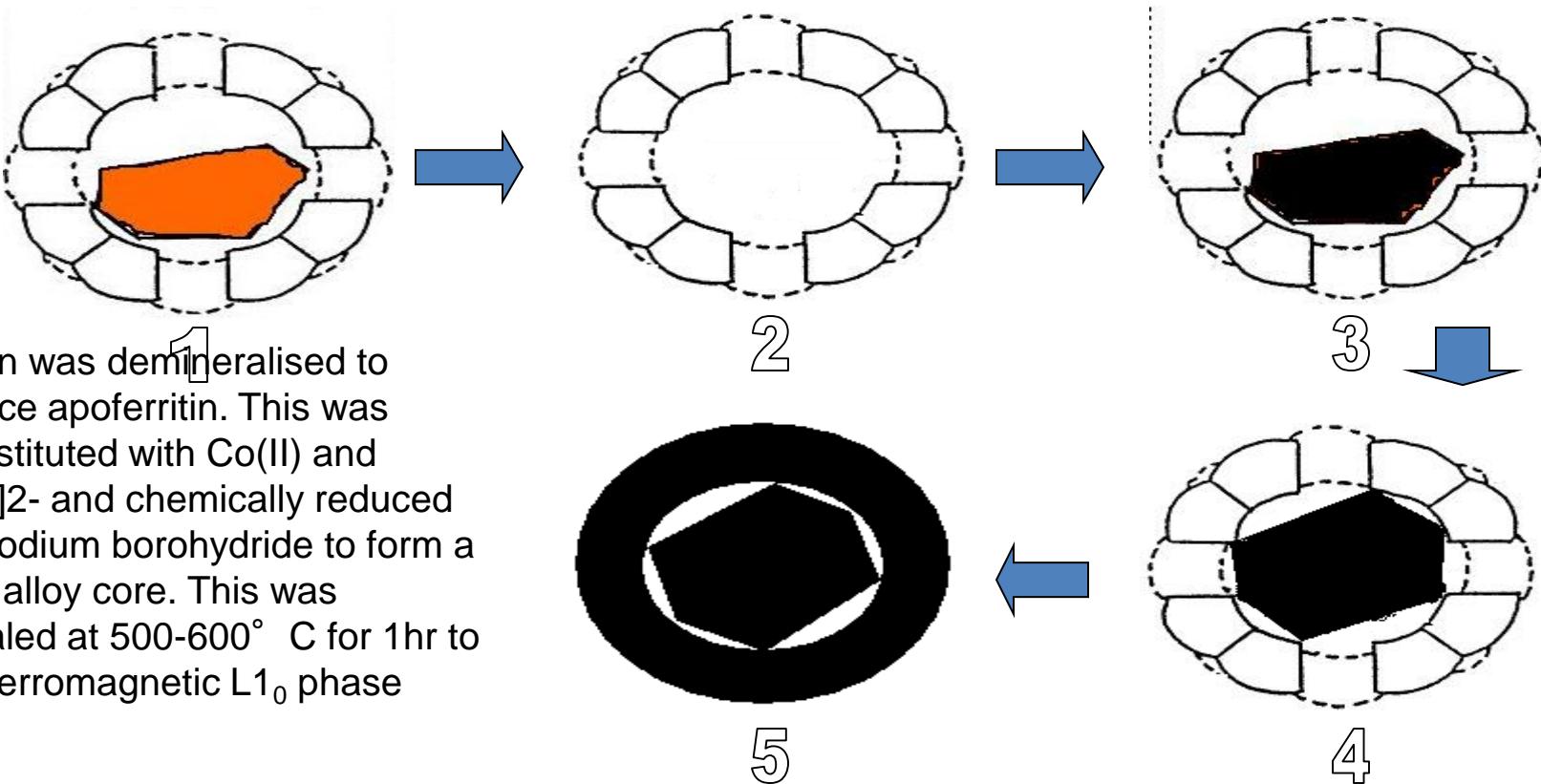
- (a) Reduction dissolution of iron-oxide core
- (b) Reconstitution of empty cage, apoferritins
- (c) Annealing



High-Density Magnetic Data Storage

- Densities of 23 Gbits cm^{-2} have been demonstrated in the laboratory using biomimetic ferritins
- Equiatomically alloyed $L1_0$ phase CoPt and FePt are being considered for ultrahigh-density recording films because of their high magnetocrystalline anisotropy that provides thermal stability for grains 3–4 nm in diameter

$L1_0$ phase CoPt Production



Method to prepare CoPt nanoparticles

Reduction and dissolution of native ferrihydrite core to form apoferritin



Reconstitution of apoferritin with Co(II) and $[PtCl_4]^{2-}$, and chemical reduction to form a metal alloy core



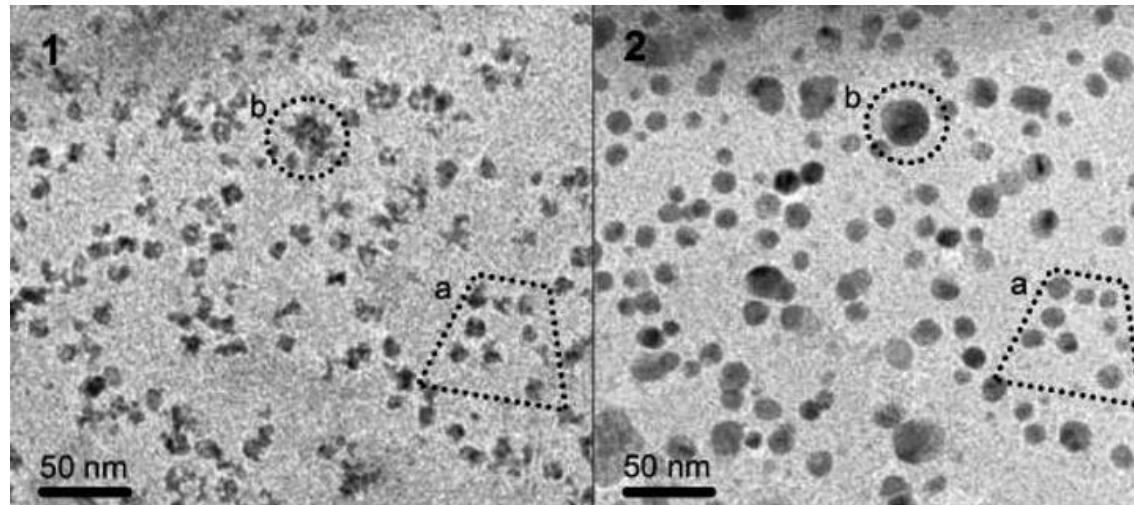
Annealing of CoPt core to produce the $L1_0$ CoPt phase encased in a carbonized matrix

$L1_0$ phase CoPt Data Storage

- CoPt highly anisotropic particles
- Size limit due to Ferritin cage
- Relatively monodisperse
- Self-organising spherical shape
- 2 nm protein shell prevents sintering
- Possibility of forming Hexagonally close-packed (HCP) arrays of $1550 \text{ G bit cm}^{-2}$

(Nano Magnetics Ltd U.K.)

Results



TEM images of a dispersion of biomimetic ferritin CoPt nanoparticles on a Si_3N_4 window prior to (1) and after (2) annealing at 550 °C for 60 minutes in H_2 . Particles that are obviously separated by a protein coating prior to annealing remain discrete afterwards (a), but indistinct material exhibits sintering (b).

Major Highlights.

- The composition, cleanliness and uniformity of the ferritin-derived nanomagnets, as well as control over their magnetic orientation, competitive materials are likely to reach the market in the near future.
- Biomimetic ferritins for nanoparticle synthesis, assembly, and application offers additional significant advantages compared with more conventional technologies.

Major Highlights.

- Self-assembled ferritin cages are readily available and naturally monodisperse, providing a reproducible system.
- Inherent biocompatibility and conjugation properties of the external surface of the protein cage make these bioinorganic magnetic ferritins ideal for pharmaceutical and medical applications such as **contrast agents, targeted drug delivery, and immunomagnetic labelling.**
- Biologically targeted semiconducting nanoparticles can also be used for **rapid protein or DNA sequencing.**

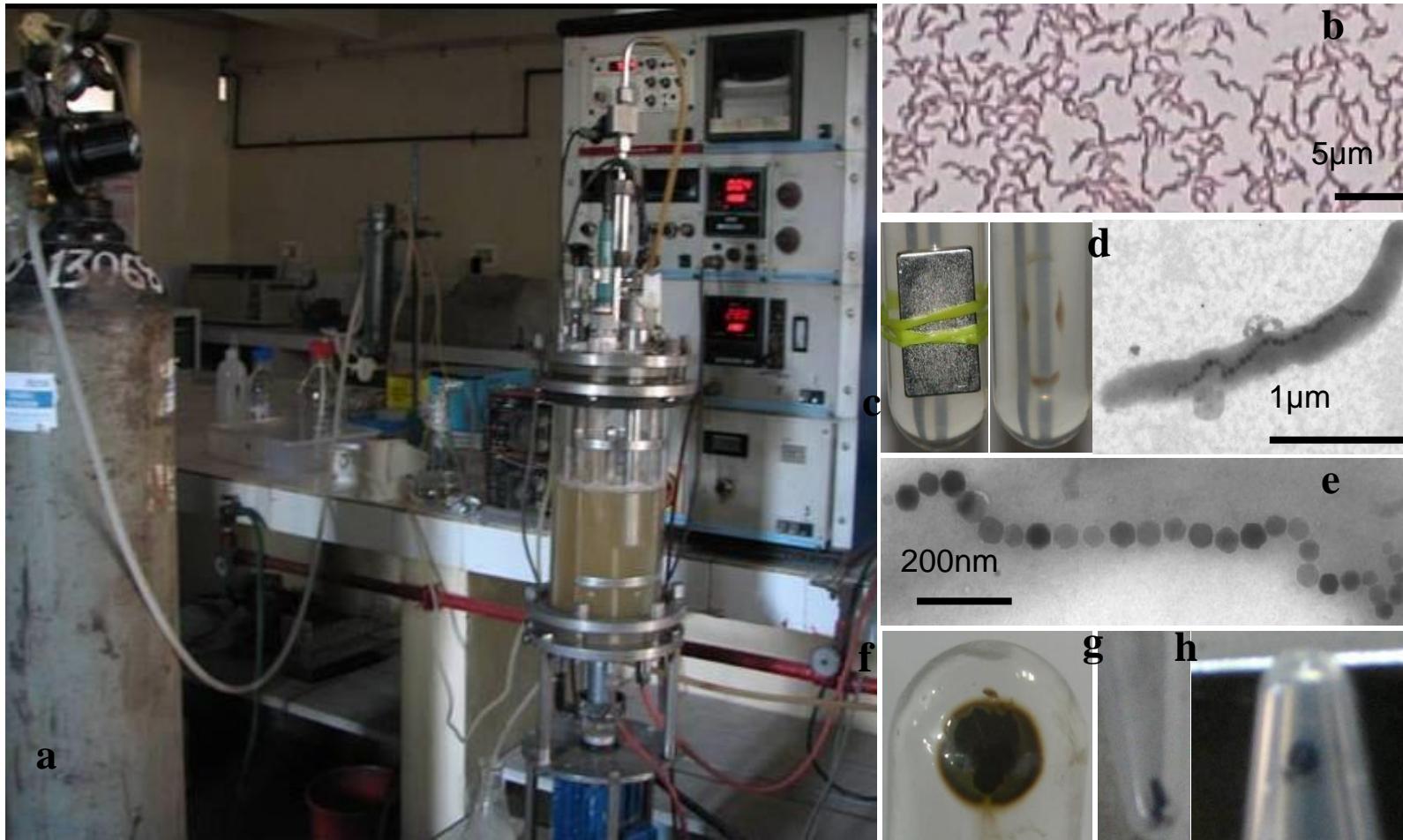
Magnetosomes: Nanoscale Magnetic Iron Minerals in Bacteria

Magnetotactic Bacteria

- Magnetotactic bacteria found in chemically stratified water columns or sediments where they occur predominantly in or below the **microaerobic redox transition zone**, between the aerobic zone of upper waters or sediments and the anaerobic regions of the habitat.
- Magnetotaxis is the orientation and navigation along magnetic field lines by motile, aquatic, bacteria
- They are a **diverse group of microorganisms** with respect to morphology, physiology, and phylogeny
- Common morphotypes include **coccoid to ovoid cells, rods, vibrios, and spirilla** of various dimensions, and multicellular forms.
- All Magnetotactic bacteria are **motile** by means of flagella
- Possess a cell-wall structure characteristic of Gram-negative bacteria.
- The arrangement of flagella varies between species/strains: cells with polar or bipolar single flagella and others with bundles of flagella have been observed

Magnetotactic Bacteria

- Magnetotactic bacteria are **difficult to isolate and grow in pure culture.**
- Most cultured strains belong to the genus *Magnetospirillum*
- Other species of **cultured magnetotactic bacteria** include a number of incompletely characterized organisms:
 - marine vibrios, strains MV-1 and MV-2
 - marine coccus, strain MC-1
 - marine spirillum, strain MV-4
 - *Desulfovibrio magneticus*, strain RS-1 (anaerobic, sulfate-reducing, rod-shaped bacterium)
- These cultured organisms, except strain RS-1, are obligate or facultative microaerophile
- and all are chemo organo heterotrophic, although the marine strains also grow chemo litho autotrophically.



Magnetosomes

- All magnetotactic bacteria contain magnetosomes
- **Magnetosomes are intracellular structures comprising magnetic iron mineral crystals enveloped by a membrane vesicle.**
- The **magnetosome membrane (MM)** is presumably a structural entity that **anchors the crystals at particular locations** in the cell,
- MM is locus of **biological control over the nucleation and growth** of the magnetosome crystals.
- The MM in genus *Magnetospirillum* is a **lipid bilayer consisting of neutral lipids, free fatty acids, glycolipids, sulfolipids, and phospholipids**
- It is often located **adjacent to the cytoplasmic membrane**, although there is no clear microscopic evidence for direct connections between the two.
- Empty and partially-filled vesicles have been reported in iron-starved cells, suggesting that **magnetosome vesicles are formed prior to the deposition of the mineral crystals**

Magnetosomes

- The magnetosome **magnetic mineral phase consists of magnetite, Fe_3O_4 , or greigite, Fe_3S_4 .**
- Each magnetotactic species or strain exclusively produces either magnetite or greigite magnetosomes, except for one marine organism that produces magnetosomes of both kinds
- The magnetosome **crystals are 35 to 120 nm in length**, although crystals with lengths of ~ 200 nm are known
- In most magnetotactic bacteria, the magnetosomes are **organized in straight chains of various lengths parallel to the long axis of the cell**
- Dispersed aggregates or clusters of magnetosomes occur in some magnetotactic bacteria, usually at one side of the cell, which often corresponds to the site of flagellar insertion
- **High degree of control over magnetosome formation and arrangement** is evident through:
 - The narrow size range of magnetosome crystals
 - Consistent morphologies of the magnetosome crystals in each species or strain
 - Consistent crystallographic orientation of the magnetosomes in chains

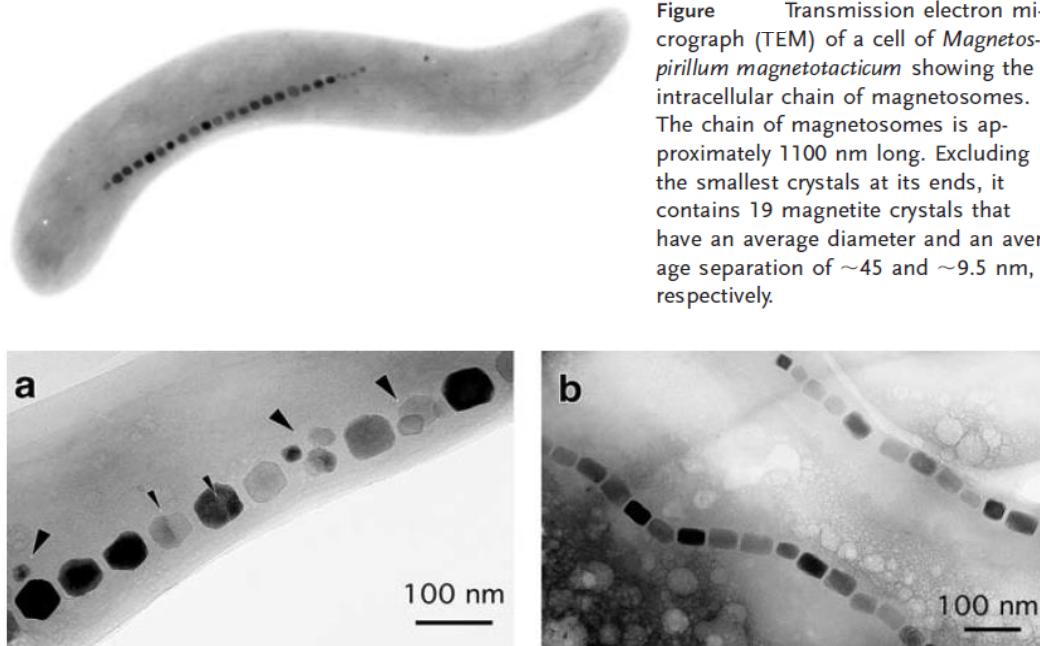


Figure Transmission electron micrograph (TEM) of a cell of *Magnetospirillum magnetotacticum* showing the intracellular chain of magnetosomes. The chain of magnetosomes is approximately 1100 nm long. Excluding the smallest crystals at its ends, it contains 19 magnetite crystals that have an average diameter and an average separation of ~45 and ~9.5 nm, respectively.

Figure Higher magnification TEMs of magnetosome magnetite crystals in (a) *M. magnetotacticum*, and (b) cultured strain MV-1. Arrows in (a) indicate crystal twinning and anomalously small crystals.

Cellular Magnetic Dipole and Magnetotaxis

- The size-range and linear arrangement of magnetosomes are highly significant for the magnetic properties of the cell
- The magnetosome crystals are within the permanent **single-magnetic-domain (SD) size-range for both magnetite and greigite**, and are thus **uniformly magnetized with maximum magnetic dipole moment per unit volume**
- Magnetic crystals **larger than SD size** are **nonuniformly magnetized** because of formation of magnetic domains or vortex configurations, further **reducing their magnetic dipole moments**.
- On the other hand, **very small SD particles are superparamagnetic (SPM)**.
- Although **SPM particles** are uniformly magnetized, their magnetic dipole moments are not constant because of **spontaneous, thermally induced, reversals which produce a time-averaged moment of zero**.
- Therefore, magnetotactic bacteria produce magnetosomes with the **optimum particle size for the maximum, permanent, magnetic dipole moment per magnetosome**.

Cellular Magnetic Dipole and Magnetotaxis

- The arrangement of the SD magnetosomes in chains maximizes the dipole moment of cell
- The reason being, magnetic interactions between the magnetosomes in a chain cause **each magnetosome moment to orient spontaneously and in parallel with others along the chain axis, minimizing the magnetostatic energy.**
- Thus the total dipole moment of the chain, **M, is the algebraic sum of the moments of the individual magnetosomes in the chain**
- However, this is true **only because magnetosomes are physically constrained by magnetosome membranes** in the chain configuration.
- If free to float in the cytoplasm, magnetosomes would likely clump, resulting in a much smaller net dipole moment than in the chain.
- For organisms such as *Magnetospirillum magnetotacticum*, the remanent moment is the maximum possible moment of the chain

Cellular Magnetic Dipole and Magnetotaxis

- Magnetotaxis results from passive orientation of a swimming magnetotactic bacterium along local magnetic field by torque exerted by the field \mathbf{B} (e. g., the geomagnetic field) on cellular dipole moment \mathbf{M}
- A chain of 10–20 magnetosomes, each of dimension 50 nm, would be sufficient for the orientation of a magnetotactic bacterium in the geomagnetic field at ambient temperature.
- Since the chain of particles is fixed within the cell, **the entire cell is oriented by torque exerted on magnetic dipole by the magnetic field.**
- **If the magnetic field is decreased,** the time-averaged orientation of the cell along the field is decreased and the migration rate of the cell along the magnetic field direction is decreased, even though the **forward-swimming speed of the cell is unchanged.**
- Thus, magnetotactic bacteria behave like **self-propelled magnetic dipoles.**

Cellular Magnetic Dipole and Magnetotaxis

- The potential energy (E) of the cellular magnetic dipole moment in the magnetic field is given by:

$$E_\Theta = - MB \cos\Theta \quad (1)$$

where Θ is the angle between \mathbf{M} and \mathbf{B} .

- Thermal energy at ambient temperatures will tend to cause misalignment of the swimming bacterium.
- In thermal equilibrium at temperature T , the probability of the moment having energy E_Θ is proportional to the Boltzmann factor $\exp(-E_\Theta/kT)$, where k is Boltzmann's constant.
- The thermally averaged projection of the dipole moment on the magnetic field $\langle \cos\Theta \rangle$ can be determined from the Langevin theory of paramagnetism and is given by Langevin function $L(\alpha)$

$$\langle \cos\Theta \rangle = L(\alpha) = \coth(\alpha) - 1/\alpha \quad (2)$$

where $\alpha = MB/kT$. $L(\alpha) = 0$ for $\alpha = 0$ and asymptotically approaches 1 as α approaches ∞ .

- In particular, $L = 0.9$ when $\alpha = 10$.

Cellular Magnetic Dipole and Magnetotaxis

- Experimental determination of the **average dipole moment per cell** of *M. magnetotacticum* by electron holography gave a value of $5 \times 10^{16} \text{ Am}^2$
- In geomagnetic field of 50 μT at room temperature, $L(\alpha)$ is greater than 0.8, meaning that migration rate of cells along the local direction of the geomagnetic field would be 80% of their forward-swimming speed.
- If the number of magnetosomes, and hence \mathbf{M} , is low, then the alignment of the cell and its migration along the field lines is inefficient.
- On the other hand, increasing the number of magnetosomes beyond a certain value will not significantly improve the alignment of the cell in the field because of the asymptotic approach of $L(\alpha)$ to 1 for large α .
- Magnetotactic bacteria control the biomineralization process to produce a sufficient number of magnetosomes of optimal size for efficient magnetic navigation in the geomagnetic field.

Magneto-Aerotaxis

- Like other free-swimming bacteria, magnetotactic bacteria propel themselves through the water by rotating their helical flagella.
- Because of their magnetosomes, magnetotactic **bacteria are passively oriented and actively migrate along the local magnetic field B** , which in natural environments is the geomagnetic field.
- When distinct morphotypes of magnetotactic bacteria, isolated and grown in pure culture, were studied in oxygen concentration gradients using thin, flattened capillaries (Vitrocom, Inc.), it became clear **that magnetotaxis and aerotaxis work together in these bacteria**
- The behavior observed in these strains referred to as “magneto-aerotaxis”, and two different magneto-aerotactic mechanisms – termed polar and axial – are found in different bacterial species.

Magneto-Aerotaxis

- For both polar and axial magnetotactic bacteria, the **cellular magnetic dipole remains oriented along the local magnetic field, but direction of migration along magnetic field lines is determined by sense of flagellar rotation, which in turn is controlled by aerotactic receptors.**
- **Magnetotactic bacterium is essentially a self-propelled magnetic dipole with an oxygen sensor.**
- Magnetotaxis effectively turns a three dimensional search problem to find the optimal oxygen concentration into a one-dimensional search problem by using the magnetic field.

Magnetite Crystals in Magnetosomes

- HR-TEM, selected-area electron-diffraction studies, and electron holography revealed that **magnetite crystals within magnetotactic bacteria are of high structural perfection** and have been used to determine their idealized morphologies
- The morphologies are all derived from **combinations of {111}, {110}, and {100} forms** (a form refers to the equivalent symmetry related lattice planes of the crystal structure) with some distortions
- These include cuboctahedral ($[100] + [111]$), and elongated, non equidimensional prismatic
- The **cuboctahedral crystal morphology preserves symmetry of face-centered cubic spinel structure** that is, all symmetry-related crystal faces are equally developed.
- In the elongated and prismatic morphologies, some symmetry-related faces are unequally developed.
- This implies anisotropic growth conditions, for example, an anisotropic ion flux into the magnetosome membrane.
- It is thought that magnetite forms from a precursor, an amorphous iron oxide phase

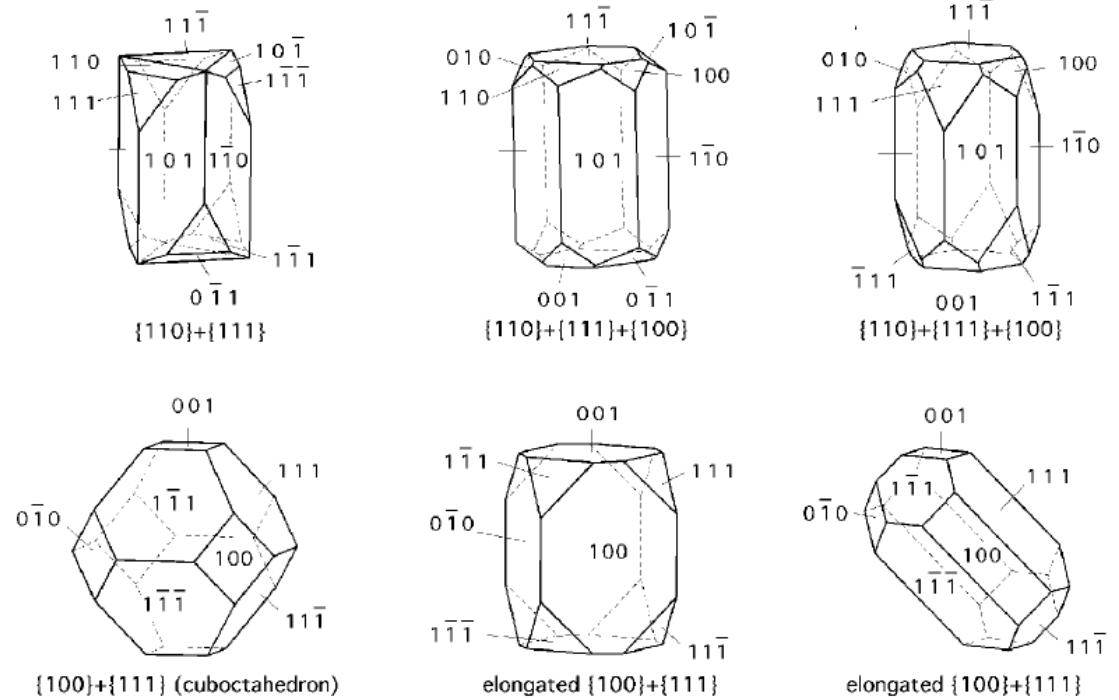


Figure Morphologies of Fd3m magnetite and greigite crystals comprising the low index forms {100}, {111}, and {110}. Anisotropic growth causes symmetry breaking in all but the cuboctahedron (lower left).

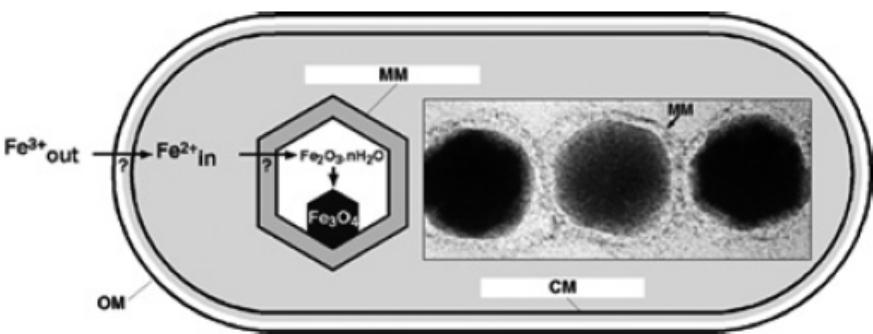
Greigite Crystals in Magnetosomes

- All freshwater, magnetotactic bacteria have been found to synthesize magnetite magnetosomes, **many marine, estuarine, and salt-marsh species produce iron sulfide magnetosomes** consisting primarily of the magnetic iron sulfide, **greigite (Fe_3S_4)**
- While **none of these organisms is currently available in pure culture**
- Recognized greigite-producing magnetotactic bacteria include **a multicellular, magnetotactic prokaryote and a variety of relatively large, rod-shaped bacteria.**
- The greigite crystals in their magnetosomes are thought to form from **nonmagnetic precursors including mackinawite (tetragonal FeS)** and possibly a **sphalerite-type cubic FeS**
- Morphologies of the greigite crystals also appear to be species and or strain-specific
- There is one reported instance of a marine bacterium that contains magnetite and greigite magnetosomes co-organized within the same magnetosome chain

Biochemistry and Gene Expression in Magnetosome Formation

- Knowledge of the biochemical and genetic controls on magnetite production is essential to understanding how the magnetotactic bacteria produce magnetosomes and organize them in chains.
- Although progress has been made by several laboratories, and the genomes of two magnetotactic bacteria (*M. magnetotacticum* and strain MC-1) have been sequenced, the overall process is not well understood and is a focal point for future work.
- **Magnetosome mineral formation must begin with transport of Fe into the cell and deposition within the MM vesicles to form a saturated Fe solution**
- Manipulation of the redox conditions within the MM vesicles so that $[\text{Fe(III)}]/[\text{Fe(II)}]$ was ~ 2 , corresponding to a redox potential of about -100 mV at elevated pH, would make magnetite the most stable Fe-oxide phase
- The MM could also provide sites for nucleation and growth of the magnetite crystals.
- Interactions of the MM with the faces of the growing crystal could affect crystal morphology

Figure Model for magnetosome mineral formation in the magnetosome membrane vesicles in magnetotactic bacteria. CM, cytoplasmic membrane; MM, magnetosome membrane; OM, outer membrane. (Adapted from a figure kindly provided by D. Schüler.)



Biochemistry of Magnetosome Formation

- Schüler and Baeuerlein found that **initially Fe-starved cells became magnetic (i. e., formed magnetosomes) within ~ 10 minutes after incubation in 10 mM Fe(III)**.
- Since Fe can amount to 2–3% of the dry weight of a magnetotactic bacterium, **an efficient Fe uptake system is required**.
- An important question in the magnetite synthesis process concerns whether Fe(III) or Fe(II) is transported into magnetosome membrane vesicles.
- Cells of *Magnetospirillum gryphiswaldense* mainly **take up Fe(III) via a high-affinity system**
- **Magnetotactic bacterial cells have Fe(III) reductase activity**, and a Fe(III) reductase has been isolated from *M. magnetotacticum*.
- Fe(III) reductase appears to be cytoplasmic, it may be **bound on the inner side of the cytoplasmic membrane and could participate in the reduction of Fe(III) as it enters the cell**

Gene Expression in Magnetosome Formation

- Since the **MM is thought to be of paramount importance in magnetosome mineral formation**, researchers have focused on the **role MM proteins** – that is, proteins which occur in the magnetosome membrane, in magnetosome synthesis
- Two different approaches have been used:
 - N-terminal amino acid sequencing of the MM proteins, followed by “reverse genetics” to obtain gene sequences for these proteins
 - Performing biochemical protein comparison of mutants that do not produce magnetosomes with wild-type strains, then again using “reverse genetics” to determine gene sequences

Gene Expression in Magnetosome Formation

| Organism | Gene | Function/homology to existing proteins |
|--|--------------|---|
| <i>M. gryphiswaldense</i> and <i>M. magnetotacticum</i> | MamA | tetratricopeptide repeat proteins |
| | MamB | cation diffusion facilitators |
| | MamE | HtrA-like serine proteases |
| <i>M. magneticum</i> strain AMB-1 | <i>MagA</i> | proton-gradient-driving H/Fe(II) antiporter |
| <i>Magnetospirillum</i> strain AMB-1 and <i>M. magnetotacticum</i> | <i>mms6</i> | acyl-CoA transferase |
| | <i>mms16</i> | GTPase activity, also involved in MM vesicle formation by invagination and budding from the cytoplasmic membrane |
| | <i>mpsA</i> | the most abundant of the three, is apparently bound to magnetite and may function in regulation of crystal growth |

Applications of Magnetosomes

- Magnetosomes have been exploited in a number of applications
- Commercial applications include
 - Immobilization of enzymes for use in biosensors,
 - Formation of magnetic antibodies in various fluoro immunoassays
 - quantification of IgG
 - Detection and separation of various cell types
 - Transfer of genes into cells.
- Magnetosomes also show promise as MRI contrast enhancement agents.
- Whole magnetotactic bacteria have been used for cell separations, as oxygen biosensors, and in studies of magnetic domains in meteorites and terrestrial rocks.

Research Methods

- Research on magnetosomes in magnetotactic bacteria primarily involves conventional, well-known, microbiological and molecular biological methods.
 - Details on culturing magnetotactic bacteria,
 - Extracting magnetosomes
 - Analyzing MM proteins and genes

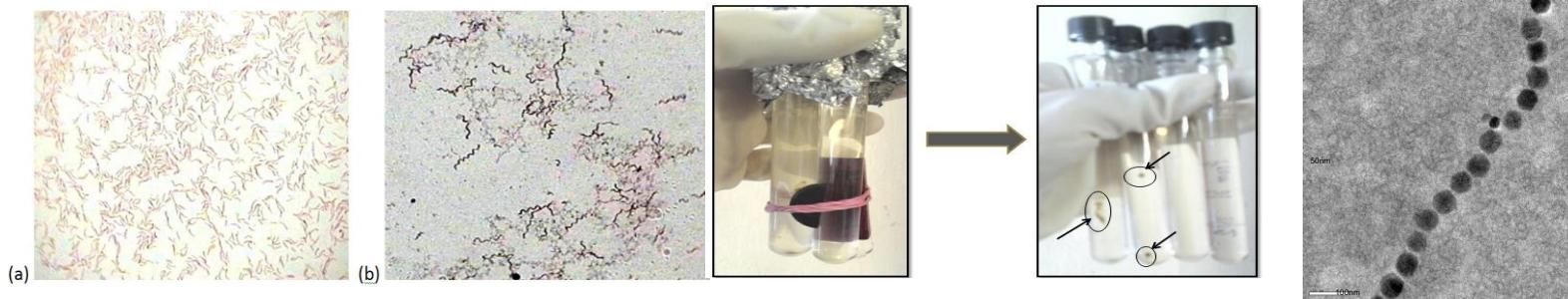
Conclusion

- Magnetotactic bacteria have solved the problem of constructing an **internal, permanent, magnetic dipole that is sufficiently robust so that a cell will be aligned along the geomagnetic field as it swims**, yet be no longer than the length of the cell (ca. 1–2 mm).
- The solution involves a **hierarchical structure – the magnetosome chain – and a mineralization process in which the mineral type, grain size and placement in the cell are all controlled by the cell**.
- Primary control is presumably exerted by the magnetosome membrane through MM proteins.
- The roles of the MM proteins and the details of the magnetosome mineralization process are the most important issues to be elucidated.

Future Research Directions

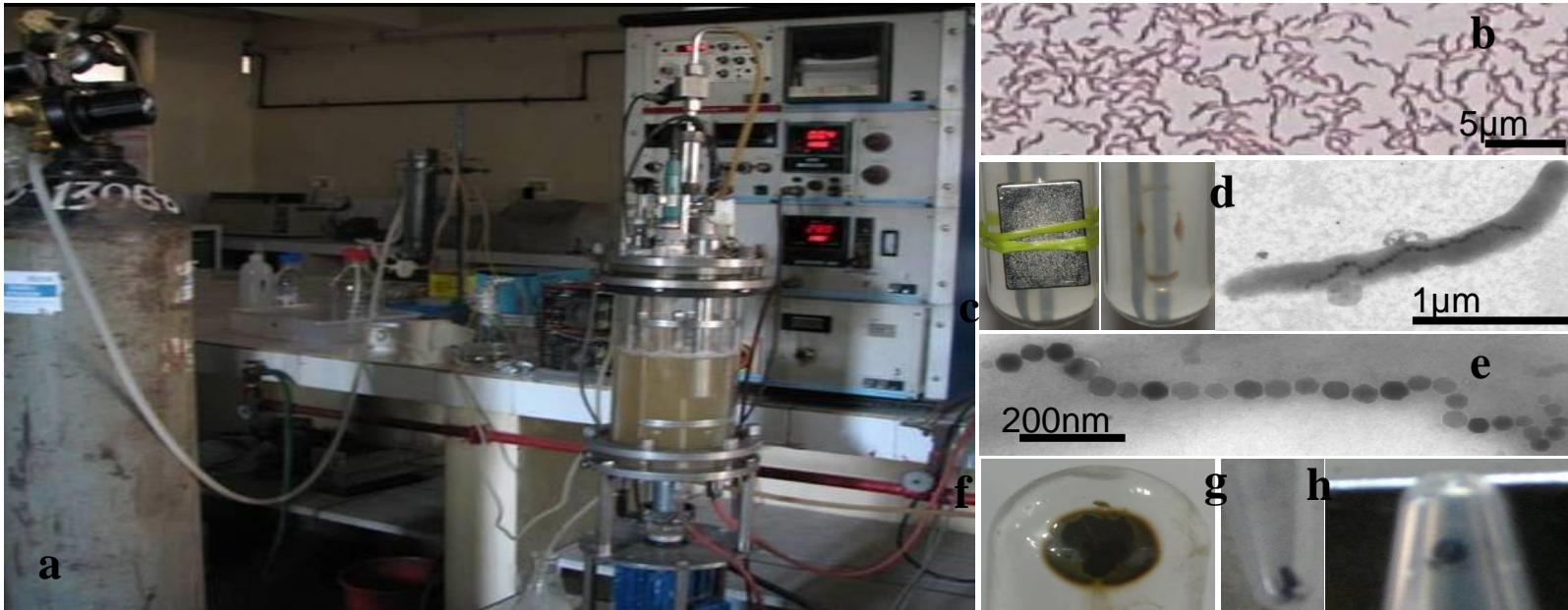
- Directions for future research include the development of genetic systems in order to move genes in and out of magnetotactic bacteria, and to **prepare knock-out mutants of strains in order to determine the functions of the MM proteins in magnetosome mineralization.**
- It would be useful **to develop convenient assays for the MM proteins.**
- It would also be useful to develop methods for determining the mineral intermediates of magnetite and greigite mineralization and quantifying them over time.
- Methods need to be devised for culturing bacteria with greigite magnetosomes, in order to compare the biomineralization processes for greigite and magnetite.

*Magnetotactic bacteria (*Magnetospirillum gryphiswaldenes*) for production of bionano magnets*



MTB culture grown in HS medium (ii) Gram staining (a) Shorter and wider after culture revival (b) Longer and slender bacteria after repeated culturing (c) Response to magnet (d) HR-TEM of magnetosomes

Growth and characterization of *M. gryphiswaldense* and magnetosome formation



a- Cultivation of MB in 3 L fermentor. b and c – Morphological and magnetic characterization of MB, d – TEM image of MB, f – cell pellet of Magnetic MB, g – lysed inorganic Fe₃O₄ crystals, h Response to extrernal magnetic field

ARTICLE**BIOTECHNOLOGY
AND
BIOENGINEERING****The Chemical Formula of a
Magnetotactic Bacterium**Mohit Naresh,¹ Sayoni Das,¹ Prashant Mishra,¹ Aditya Mittal²¹Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India²Kusuma School of Biological Sciences, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India; telephone: 011-91-11-26591052; fax: 011-91-11-26582037; e-mail: amittal@bioschool.iitd.ac.in

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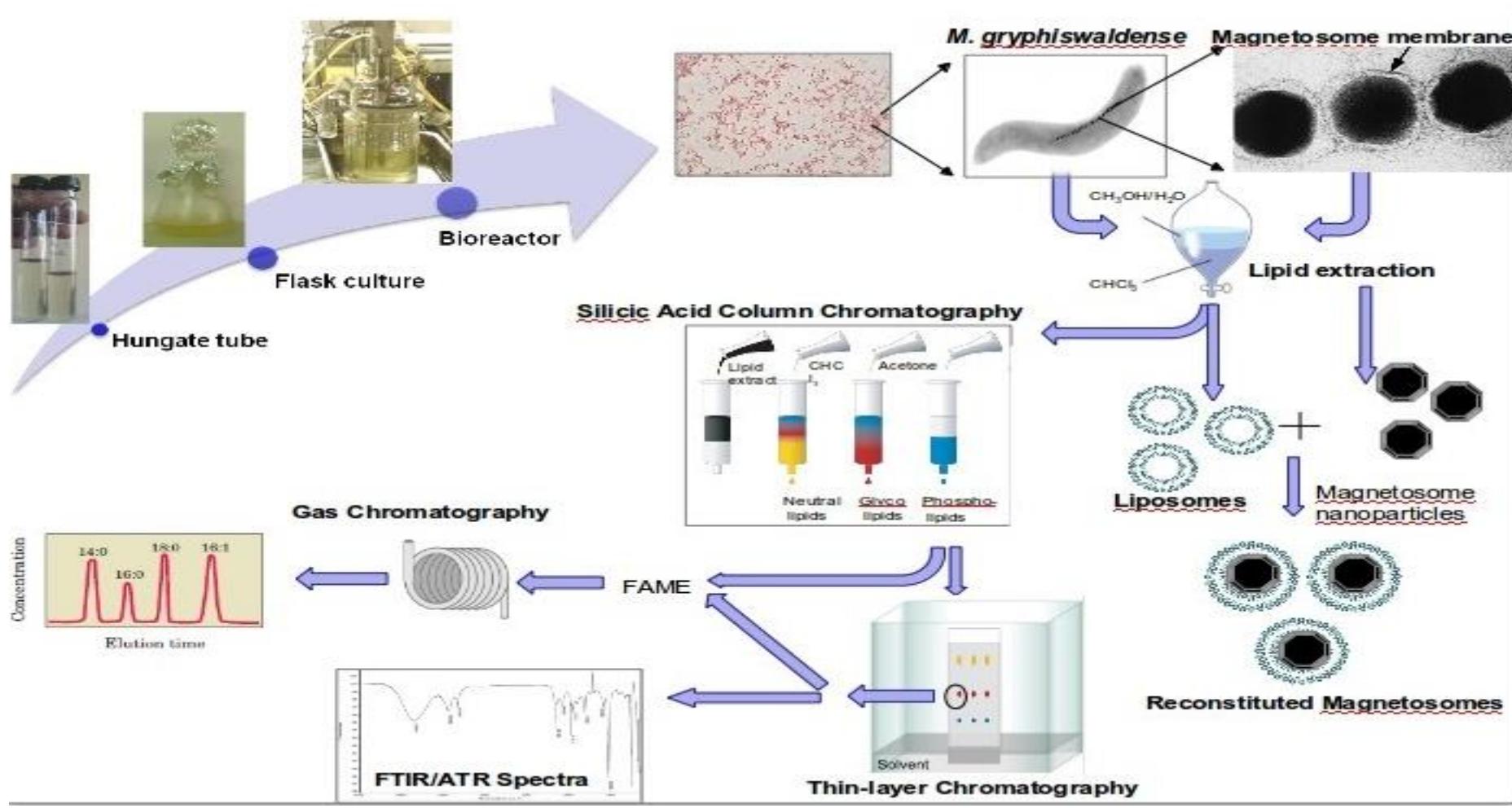
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ABSTRACT: Elucidation of the chemical logic of life is one of the grand challenges in biology, and essential to the progress of the upcoming field of synthetic biology. Treatment of microbial cells explicitly as a “chemical” species in controlled reaction (growth) environments has allowed fascinating discoveries of elemental formulae of a few species that have guided the modern views on compositions of a living cell. Application of mass and energy balances on living cells has proved to be useful in modeling of bioengineering systems, particularly in deriving optimized media compositions for growing microorganisms to maximize yields of desired bio-derived products by regulating intra-cellular metabolic networks. In this work, application of elemental mass balance during growth of *Magnetospirillum gryphiswaldense* in bioreactors has resulted in the discovery of the chemical formula of the magnetotactic bacterium. By developing a stoichiometric equation characterizing the formation of a magnetotactic bacterial cell, coupled with rigorous experimental measurements and robust calculations, we report the elemental formula of *M. gryphiswaldense* cell as $\text{CH}_{2.06}\text{O}_{0.1}\text{N}_{0.26}\text{Fe}_{1.74} \times 10^{-4}$. Remarkably, we find that iron metabolism during growth of this magnetotactic bacterium is much more correlated individually with carbon and nitrogen, compared to carbon and nitrogen with each other, indicating that iron serves more as a nutrient during bacterial growth rather than just a mineral. Magnetotactic bacteria have not only invoked some interest in the field of astrobiology for the last two decades, but are also prokaryotes having the unique ability of synthesizing membrane

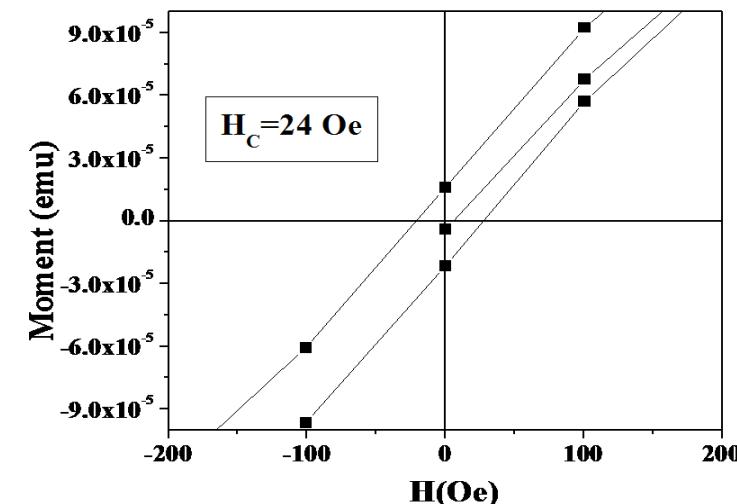
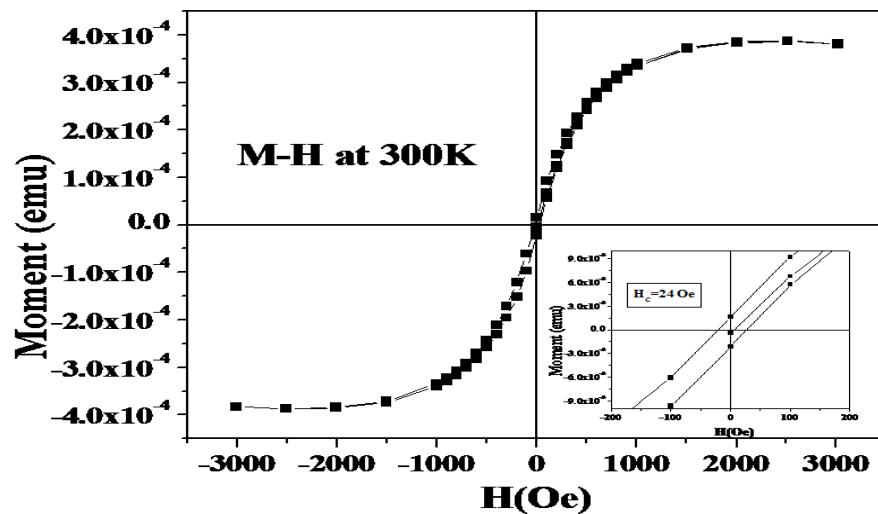
KEYWORDS: magnetosome; biominerization; synthetic biology; bioprocess; calculations; biosynthesis

Introduction

Magnetotactic bacteria, discovered by Blakemore (1975), are aquatic prokaryotes which grow at ambient/mesophilic conditions. These bacteria have a unique feature of consuming soluble salts of iron from growth media to form intracellular chains of single magnetic crystals (30–40 nm) of magnetite (Fe_3O_4) and greigite (Fe_3S_4) (Bazylinski et al., 1995; Blakemore et al., 1979; Fairev et al., 2007; Fairev and Schüller, 2008; Mann et al., 1990). These intracellular nano-crystals, each encapsulated by its own biological membrane about 3–4 nm thick (Gorby et al., 1988; Grunberg et al., 2004) are known as magnetosomes. The bio-derived magnetosomes are eco-friendly, non-toxic, and exhibit high degree of uniformity for crystals shape, size, and orientation (elegantly discussed in Fairev and Schüller, 2008; and see Naresh et al., 2009, 2011 for specific examples). Further,



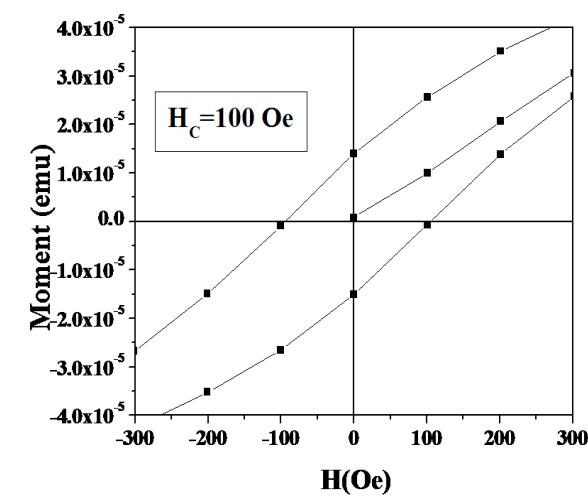
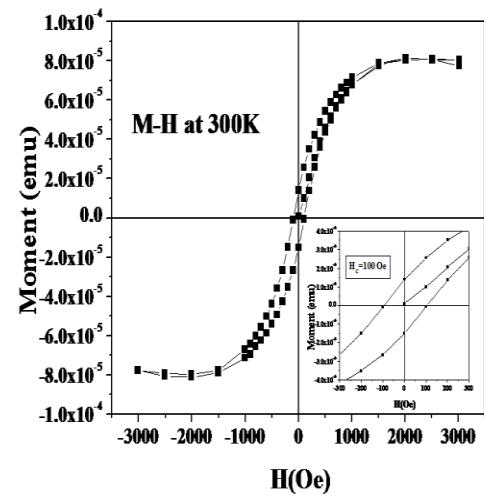
Squid (Super conducting quantum interference device) Measurements M-H curve of Bacterial sample grown in the absence of Mn (Ferric Citrate)



Coercivity is a measure of the ability of a ferromagnetic material to withstand an external magnetic field without becoming demagnetized.

Coercivity (H_c) Intensity of applied magnetic field required to reduce magnetization to zero

Squid Measurement M-H curve of Bacterial sample grown in the presence of Mn (MnCl_2)





Microbial Nanoparticle Production

Bionanotechnology
BBL747

Introduction

- **Inorganic materials in the form of hard tissues** are an integral part of most multicellular biological systems.
- Hard tissues are generally **biocomposites containing structural biomacromolecules and 60 different kinds of minerals** that perform a variety of vital structural, mechanical, and physiological functions
- Unicellular organisms such as bacteria and algae also are capable of synthesizing inorganic materials, both intra- and extracellularly
- Examples of such organisms include **magnetotactic bacteria which synthesize magnetite particles, diatoms and radiolarians that synthesize siliceous materials, and S-layer bacteria that synthesize gypsum and calcium carbonate as surface layers**
- These bioinorganic materials can be extremely complex both in structure and function, and also exhibit exquisite hierarchical ordering from the nanometer to macroscopic length scales which has not been achieved in laboratory-based synthesis.

Introduction

- An important aspect of nanotechnology concerns the development of experimental procedures for the reproducible synthesis of nanomaterials of controlled size, polydispersity, chemical composition, and shape.
- Though solution-based chemical methods enjoy a long history dating back to work of Faraday on the synthesis of aqueous gold colloids, **increasing pressure to develop green chemistry, eco-friendly methods for nanomaterial synthesis** has resulted in researchers turning to biological organisms for inspiration.
- Biotechnological applications such as remediation of toxic metals have long employed microorganisms such as bacteria and yeast
- The **detoxification process occurring by reduction of the metal ions or by formation of insoluble complexes with the metal ion (e. g., metal sulfides) in the form of nanoparticles** is known.
- The possibility of using such microorganisms in the deliberate synthesis of nanomaterials is a recent phenomenon.

Microbial Nanoparticle Production

- An amalgamation of curiosity, environmental compulsions, and conviction that nature has evolved the best processes for synthesis of inorganic materials on nano- and macro-length scales has contributed to the development of a relatively new and largely unexplored area of research based on the **use of microbes in the biosynthesis of nanomaterials.**
- Some of the earliest reports on the accumulation of inorganic particles in microbes can be traced to the work of
 - Zumberg, Sigleo and Nagy (gold in Precambrian algal blooms),
 - Hosea and coworkers (gold in algal cells)
 - Beveridge and co-workers (gold in bacteria)
 - Aiking and co-workers (CdS in bacteria)
 - Reese and co-workers(CdS in yeast)
 - Temple and LeRoux (ZnS in sulfate-reducing bacteria)
 - Blakemore, Maratea and Wolfe (magnetite in bacteria)

Microbial Nanoparticle Production

- The use of microbes in the deliberate synthesis of nanoparticles of different chemical compositions include **bacteria for silver, gold, CdS, ZnS (sphalerite), magnetite, iron sulfide, yeast for PbS and CdS, and algae for gold**
- In all these studies, the nanoparticles are formed intracellularly, but may be **released into solution by suitable treatment of the biomass.**
- It has been shown that fungi when challenged with aqueous metal ions lead to the formation of nanoparticles both intra- and extracellularly
- Different genera of fungi have been identified for the extracellular synthesis of gold, silver, and CdS quantum dots, as well as the intracellular growth of nanocrystals of gold and silver.
- Extremophilic actinomycetes such as *Thermomonospora* sp. have also been used to synthesize fairly monodisperse gold nanoparticles extracellularly
- Yacaman and co-workers have demonstrated the growth of gold nanoparticles in sprouts, roots and stems of live alfalfa plants

Bacterial Nanoparticle Production

- Among the different microbes studied for the biosynthesis of nanoparticles, bacteria have received the most attention
- Tanja Klaus and co-workers showed that the metal-resistant bacterium, *Pseudomonas stutzeri* AG259 (originally isolated from a silver mine), when challenged with high concentrations of silver ions during culturing resulted in the intracellular formation of silver nanoparticles of variable shape
- Transmission electron microscopy (TEM) image of a *Pseudomonas stutzeri* AG259 cell with **silver particles located intracellularly, were crystalline, often form at the poles of the bacteria, and were not particularly monodisperse, ranging in size from a few nm to 200 nm**
- Most of the nanoparticles were found to be composed of elemental silver, while occasionally the formation of Ag₂S was observed
- The exact mechanism leading to the formation of intracellular silver nanoparticles in *P. stutzeri* AG259 is yet to be elucidated.

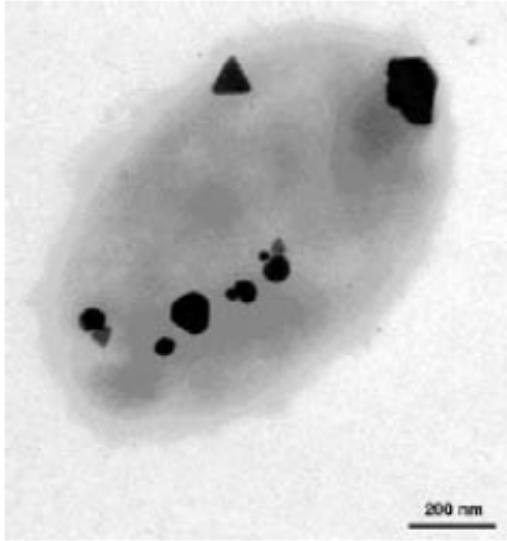


Figure Silver-based crystals with different morphology, size and chemical composition produced by *P. stutzeri* AG259. Triangular, hexagonal and spheroid Ag-containing particles are accumulated at different cellular binding sites in the periplasmic space of the bacterial cell. (Reprinted with permission from Ref. [23]; © 2000 Wiley-VCH).

Bacterial Nanoparticle Production

- Biofilms of metal nanoparticles embedded in a biological matrix may have important applications in the synthesis of **eco-friendly and economically viable cermet materials** for optically functional thin film coatings
- Jorger, Klaus and Granqvist showed that heat treatment of the Ag nano-bacteria biomass yielded hard coatings of a cermet that was resistant to mechanical scratching with a knife and whose optical properties could be tailored by varying the silver loading factor
- **The cermet material was composed primarily of graphitic carbon and up to 5% by weight (of the dry biomass) of silver.**
- In an interesting recent study, it has been demonstrated that bacteria not normally exposed to large concentrations of metal ions also may be used to grow nanoparticles
- These authors showed that *Lactobacillus* strains present in buttermilk, when exposed to silver and gold ions, resulted in the large-scale production of nanoparticles within the bacterial cells

Bacterial Nanoparticle Production

- The exposure of **lactic acid bacteria present in the whey of buttermilk to mixtures of gold and silver ions could be used to grow nanoparticles of alloys of gold and silver**
- The surface plasmon vibrations from the silver and gold bacterial colloids occur at 439 and 547 nm respectively, while for the mixed alloy case it is centered at 537 nm.
- In the case of bacteria exposed to a mixture of the metal ions, the fact that **the plasmon vibration wavelength is within the range defined by pure silver and gold nanoparticles, together with the absence of a distinct vibration corresponding to pure silver**, was argued by author to indicate the **formation of an alloy of the composition $Au_{0.75}Ag$, and not a core-shell structure**
- By using a series of time-dependent UV-visible spectroscopy and TEM measurements, it was found that **nucleation of the silver and gold nanoparticles occurs outside the bacterium** (presumably on the cell surface through sugars and enzymes in the cell wall), following which **the metal nuclei are transported into the cell where they aggregate and grow to larger-sized particles**.
- The presence of noble metal nanocenters is known to enhance Raman spectroscopic signatures, and this feature was used by the authors to probe the internal chemical environment in the bacteria

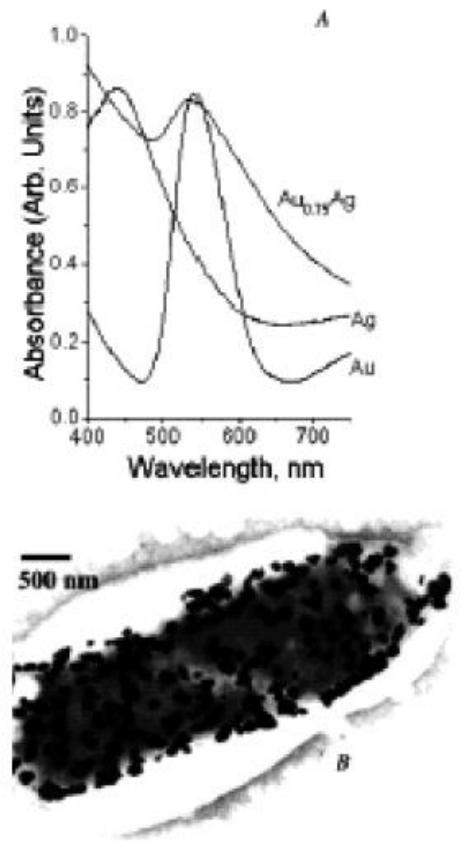


Figure (A) Comparison of the UV/visible absorption spectra of bacterial colloids of pure Au and Ag with an alloy colloid of starting composition $\text{Au}_{0.75}\text{Ag}$. The peak maxima are 547, 439, and 537 nm for Au, Ag and $\text{Au}_{0.75}\text{Ag}$, respectively. Note that there is no peak due to Ag colloid in the alloy. The spectra have been moved vertically as there is a shift in baseline from sample to sample. (B) TEM of a bacterium with alloy crystallites. [111] Zone axis was seen in the electron diffraction; smaller crystallites were also seen outside the bacterium. (Reprinted with permission from Ref. [24]; © 2002 American Chemical Society).



Microbial Nanoparticle Production (Contd.)

Bionanotechnology
BBL747

Bacterial Nanoparticle Production

- The development of protocols for the synthesis of semiconductor nanoparticles such as **CdS for application as quantum-dot fluorescent biomarkers in cell labeling**
- Simple **variation of the particle size enables tailoring of the band gap(valence and conduction band) and, consequently, the color of the quantum dots during UV-light irradiation**
- Exposure of the bacterium *Klebsiella aerogenes* to Cd²⁺ ions resulted in the intracellular formation of CdS nanoparticles in the size range 20–200 nm
- The composition of the nanoparticles formed was a strong function of buffered growth medium for the bacterium.

Bacterial Nanoparticle Production

- For the growth of magnetic nanoparticles, Roh and co-workers showed that metals such as Co, Cr, and Ni may be substituted into magnetite crystals biosynthesized in the thermophilic iron-reducing bacterium *Thermoanaerobacter ethanolicus* (TOR-39)
- This procedure led to the **formation of octahedral shaped magnetite nanoparticles in large quantities** that co-existed with a poorly crystalline magnetite phase near the surface of the cells.
- A more fundamental investigation into the assembly of **single-domain magnetite particles** harvested from the bacterium *Magnetospirillum magnetotacticum* into **folded-chain and flux-closure ring morphologies** was carried out by Philipse and Maas
- The TEM images show the magnetite particles extracted from the bacterial biomass by sonication, are **~ 4.7 nm in diameter and predominantly organized in the form of rings and, more infrequently, as linear superstructures**.

Bacterial Nanoparticle Production

- The magnetite crystals **are single domains with large magnetic moments** that, when constrained to lie on a two-dimensional surface, are responsible for the head-to-tail assembly.
- The circular structures were explained by the authors to be flux-closure rings of in-plane dipoles.
- In conventional ferrofluids, the magnetic moments of the particles are much smaller than that observed for biogenic magnetite and therefore, such linear and ring-like structures have not been observed.
- Magnetization measurements of dried magnetite particles harvested from the bacterial cells, established that the **biogenic magnetite nanoparticles are not superparamagnetic**

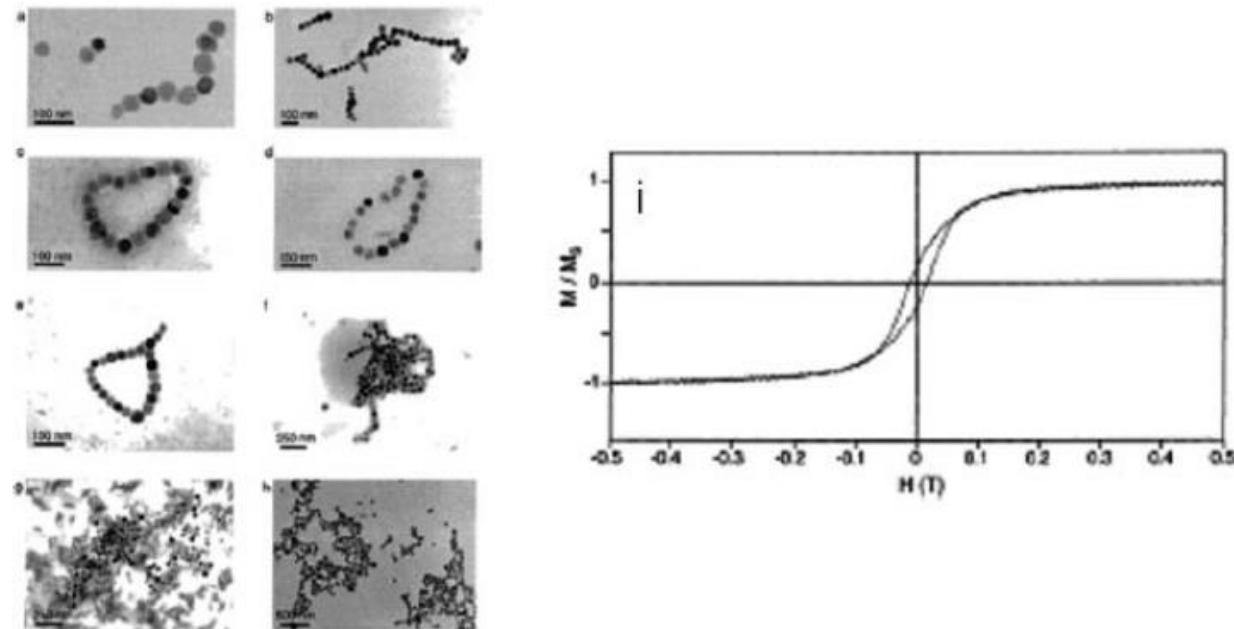


Figure Representative selection of cluster morphologies for magnetite (Fe_3O_4) colloids extracted from cells imaged by transmission electron micrographs. Note the magnetic flux closure rings in images (c), (d), and (e). The tendency to form string-like aggregates can be clearly seen in for example, images (b) and (f).

(i) Normalized magnetization M/M_S (M_S is the measured saturation magnetization at high field of dried magnetite particles extracted from cells) versus the applied magnetic field H . (Reprinted with permission from Ref. [4]; © 2002 American Chemical Society).

Fungal nanoparticles production

- Exposure of yeasts such as *Candida glabrata* and *Schizosaccharomyces pombe* to Cd²⁺ ions leads to the intracellular formation of CdS quantum dots
- In this case, Yeast cells exposed to Cd²⁺ ions produce metal-chelating peptides (glutathiones), and an increase in the intracellular sulfide concentration and the formation of nanocrystalline CdS.
- The biogenic **CdS quantum dots are capped and stabilized by the peptides, glutathione and its derivative phytochelatins with the general structure (γ -Glu-Cys)_nGly**
- The yeast, *Torulopsis* sp. as being capable of intracellular synthesis of nanoscale PbS crystallites when exposed to aqueous Pb²⁺ ions
- The PbS nanoparticles were **extracted from the biomass by freeze–thawing** and analyzed using a variety of techniques.
 - A blue shift in the absorption edge suggested that the particles were in the **quantum size regime**.
 - A HRTEM image of the PbS nanoparticles shows that the particles are spherical, size from 4 to 8 nm, crystalline and exhibit a well-developed electron diffraction pattern with evidence for mixed cubic and hexagonal phases in the particles.

Fungal nanoparticles production

- Ultimately, biogenic nanoparticles would have to compete with chemically synthesized nanoparticles in terms of performance in devices.
- As a step in this direction, Kowshik et al. have shown that **CdS quantum dots synthesized intracellularly in *Schizosaccharomyces pombe* yeast cells exhibit ideal diode characteristics**
- Biogenic CdS nanoparticles in the size range 1–1.5 nm were used in the fabrication of a heterojunction with poly(*p*-phenylenevinylene).
- Such a diode exhibited 75 mA cm⁻² current in the forward bias mode at 10 V, while breakdown occurred at 15 V in the reverse direction.
- The use of fungi in the synthesis of nanoparticles is a relatively recent addition to the list of microbes discussed above.
- A detailed screening process involving approximately 200 genera of fungi resulted in two genera which, when challenged with aqueous metal ions such as AuCl⁴⁻ and Ag²⁺, yielded large quantities of metal nanoparticles either extracellularly (*Fusarium oxysporum*) or intracellularly (*Verticillium* sp.)

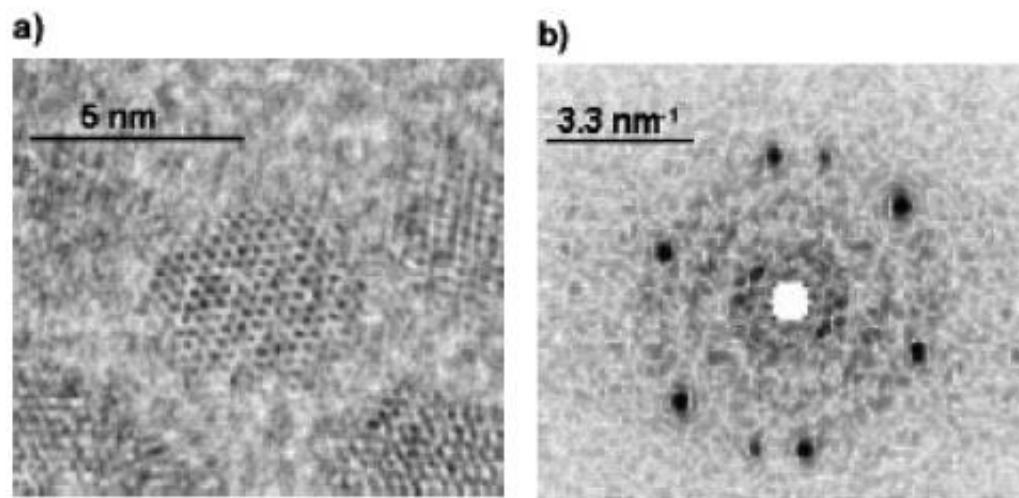


Figure (a) An HRTEM image showing the near-spherical PbS nanocrystallites; (b) their diffraction pattern. (Reprinted with permission from Ref. [35]; © 2002 Wiley-VCH).

Fungal nanoparticles production

- Flasks containing the *Verticillium* sp. biomass after exposure to 10^{-4} M HAuCl₄ solution for 72 hours have a **distinctive purple color indicating formation of gold nanoparticles** and can be seen in UV-visible absorption spectrum as a resonance at ~ 540 nm
- This resonance is clearly missing in the biomass before exposure to gold ions and in the filtrate after reaction of *Verticillium* with the gold ions.
- The gold ions are thus reduced intracellularly, further evidence of which is provided by TEM analysis of thin sections of the cells after formation of gold nanoparticles
- At low-magnification TEM image, **spherical gold nanoparticles are seen close to surface of the cells**. At higher magnification, the nanoparticles ranging in size from 5 nm to 200 nm with an average size of 20 \pm 8 nm are seen **populating both the cell wall and cytoplasmic membrane of the fungus**.
- From the powder X-ray diffraction pattern recorded from the biofilm, the gold nanoparticles are found **crystalline, and are characteristic of face-centered cubic (fcc) gold structure**.
- The reduction of the gold ions is expected to be due to reaction with enzymes present in the cell walls of the mycelia

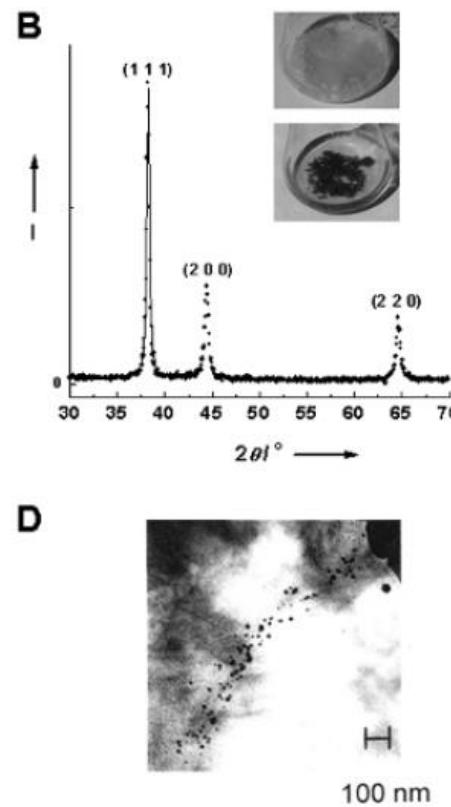
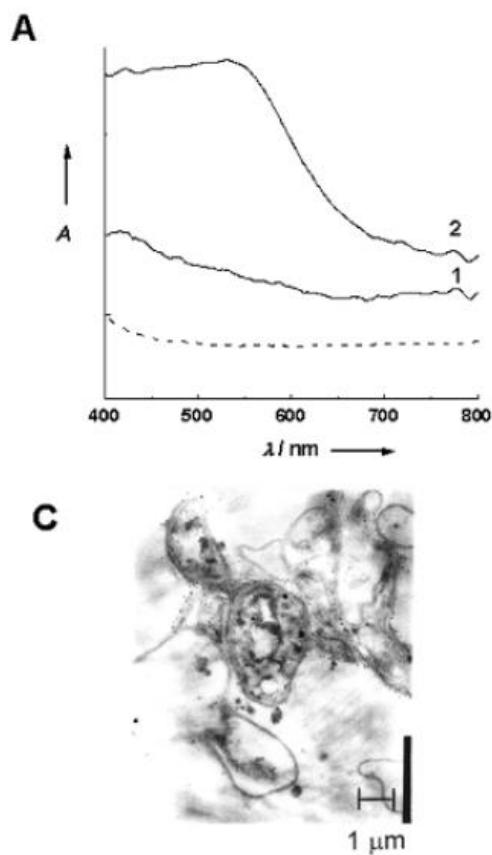


Figure (A) UV/Visible spectra recorded from biofilms of the *Verticillium* sp. fungal cells before (curve 1) and after (curve 2) exposure to 10^{-4} M aqueous HAuCl₄ solution for 72 hours. The spectrum recorded from the HAuCl₄ solution after immersion of the fungal cells for 72 hours is shown for comparison (dashed line). (B) X-ray diffraction pattern recorded from an Au nano-*Verticillium* biofilm formed on a Si (111) wafer. The principal Bragg reflections are identified. The inset shows pictures of the *Verticillium* fungal cells after removal from the culture medium (flask on top) and after exposure to 10^{-4} M aqueous solution of HAuCl₄ for 72 hours (flask at bottom). (C, D) TEM images at different magnifications of thin sections of stained *Verticillium* cells after reaction with AuCl₄⁻ ions for 72 hours.

Fungal nanoparticles production

- Exposure of *Verticillium* sp. to silver ions resulted in a similar intracellular growth of silver nanoparticles
- From the application point of view, extracellular synthesis of nanoparticles would be more important.
- It was observed that **exposure of the fungus *Fusarium oxysporum* to aqueous gold and silver ions leads to the formation of fairly monodisperse nanoparticles in solution**
- Even more exciting was the finding that **exposure of *Fusarium oxysporum* to aqueous CdSO₄ solution yielded CdS quantum dots extracellularly**
- CdS nanoparticles formed after reaction of 10⁻⁴ M CdSO₄ solution with the *Fusarium oxysporum* biomass for 12 days.
- The particles are reasonably monodisperse, and range in size from 5 to 20 nm.
- X-ray diffraction analysis of a film of the particles formed on a Si (111) wafer clearly showed that the particles were nanocrystalline with Bragg reflections characteristic of hexagonal CdS

Fungal nanoparticles production

- Reaction of the fungal biomass with aqueous CdNO₃ solution for an extended period did not yield CdS nanoparticles, indicating the possibility of release of a sulfate reductase enzyme into solution.
- Polyacrylamide gel electrophoresis (PAGE) results of the aqueous extract exposed to the fungal biomass for 12 days, indicate the presence of at least four protein bands in the extract.
- Reaction of the protein extract after dialysis (using a dialysis bag with 3kDa molecular weight cutoff) with CdSO₄ solution did not yield CdS nanoparticles.
- However, addition of ATP and NADH to the dialysate restored the CdS formation capability of the protein extract.
- It is believed that the same proteins are also responsible for the reduction of gold and silver ions
- The gold, silver, and CdS nanoparticles were stable in solution for many months due to stabilization by surface-bound proteins

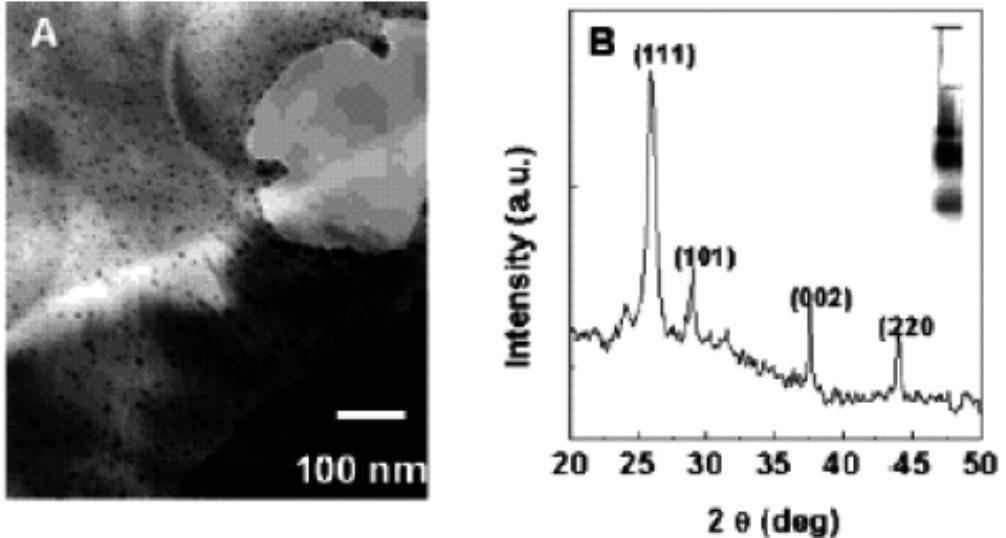


Figure (A) Bright-field TEM image of CdS nanoparticles formed by reaction of CdSO_4 with the fungal biomass for 12 days. (B) X-ray diffraction pattern recorded from the CdS nanoparticle film deposited on a Si (111) wafer. The inset shows the

native gel electrophoresis of aqueous protein extract obtained from *Fusarium oxysporum* mycelia; 10% (w/v) polyacrylamide slab gel, pH 4.3 (Reprinted with permission from Ref. [40]; © 2002 American Chemical Society).

Fungal nanoparticles production

- The development of a rational nanoparticle biosynthesis procedure using specific enzymes secreted by fungi in both the intra- and extracellular synthesis of nanoparticles has many attractive associated features.
- Plant pathogenic fungi produce copious quantities of enzymes, are usually nonpathogenic to humans, and are easily cultured in the laboratory.

Outlook

- A case for the serious investigation of microorganisms such as bacteria, algae, yeasts, actinomycetes, and fungi as possible **inorganic nanofactories** has been made.
- A number of issues from the nanotechnology and microbiology points of view require to be addressed before such a biosynthesis procedure can compete with existing physical and chemical synthesis protocols.
 - The elucidation of biochemical pathways leading to metal ion reduction or formation of insoluble complexes in the different classes of microbes is essential in order to develop a rational microbial nanoparticle synthesis procedure.
 - An understanding of the surface chemistry of the biogenic nanoparticles (i. e., the nature of capping surfactants/peptides/proteins) would be equally important.
- This would then lead to the **possibility of genetically engineering microbes to overexpress specific reducing molecules and capping agents, thereby controlling not only the size of the nanoparticles but also their shape.**

Outlook

- The rational use of **constrained environments within cells such as the periplasmic space and cytoplasmic vesicular compartments (e. g., magnetosomes) to modulate nanoparticle size and shape** is an exciting possibility.
- The range of chemical compositions of nanoparticles currently accessible by microbial methods is currently extremely limited and confined to metals, some metal sulfides and iron oxide.
- Extension of the protocols to enable reliable synthesis of nanocrystals of other oxides (TiO_2 , ZrO_2 , etc.) and nitrides, carbides, etc. could make microbial synthesis a commercially viable proposition.
- Equally intriguing are questions related to the metal ion reduction/reaction process in cellular metabolism, and whether the nanoparticles formed as byproducts of the reduction process have any role to play in cellular activity (e. g., magnetite in magnetotactic bacteria).
- Plant organisms (e. g., fungi) are not normally exposed to high concentrations of metal ions such as Cd^{2+} , AuCl_4^- and Ag^{2+} , but, when challenged, they secrete enzymes that are capable of metal ion reduction and indeed conversion of sulfates to sulfides suggests that evolutionary processes may be at play.

Bacteriorhodopsin (Part - I)

Overview and
Functions

Introduction

- Production of biological macromolecules for technical processes is “**state-of-the-art technology**”.
- The **biosynthetic** capabilities of cells go far beyond organic chemistry.
- Materials such as functional biomolecules, enzymes, antibodies, and hormones are indispensable in the food industry, cleaning, medical diagnosis, pharmacy, and therapy etc.
- So companies for DNA chips, readers, high throughput screening were established

Need for new Technology

- Need for miniaturization is increasing challenges for lithographic methods
- The utilization of self-organizing principles and bioengineering of functional biological structures are promising alternate
- Though re-engineering of biomolecules for technically desirable function has become possible
- Problem is communication between classical microsystem (electronic) and nanoscaled biomolecules
- Interface remains major challenge in realization of cross-technology products.
- Stability of biomaterials compared to organic and semiconductor material is another challenge.

Overview : The Molecular Properties of Bacteriorhodopsin

- Bacteriorhodopsin has been studied over the past two decades as a material for technical applications.
- It is very stable at high temperature.
- It has several technically interesting functions
- Tools for both its modification and production in technical quantities have been developed, and
- It offers various interface principles, whether optical, electrical, or chemical.

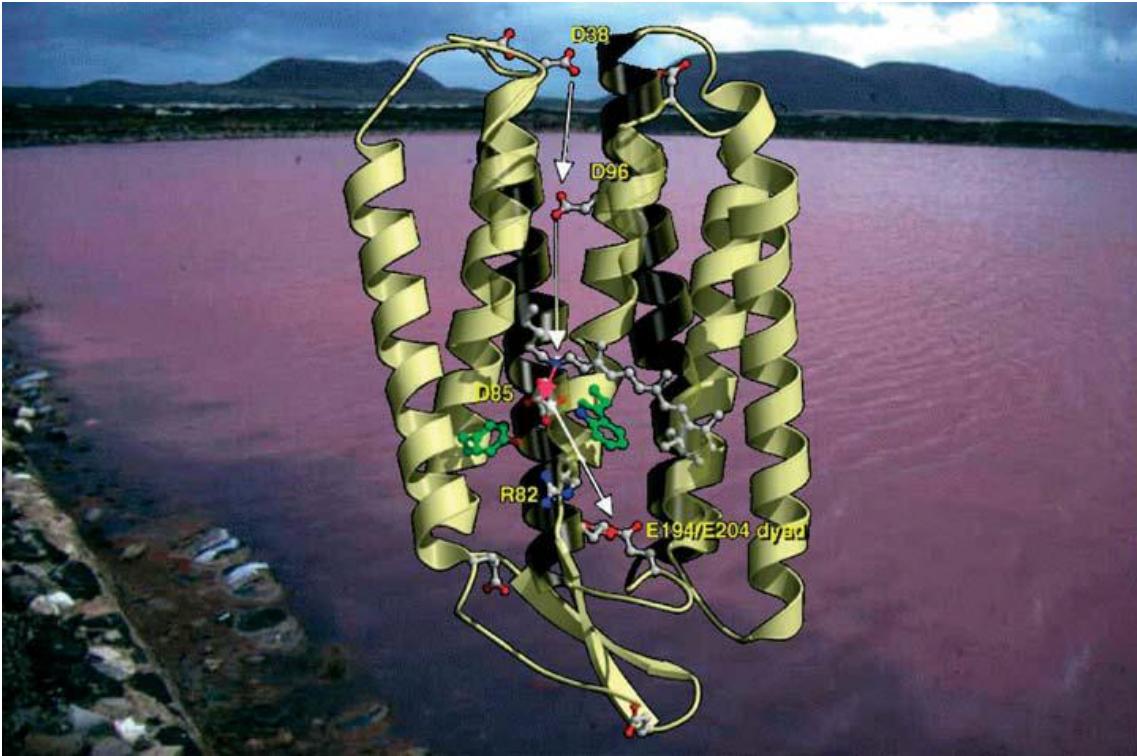
Haloarchaea and their Retinal Proteins

- Archaea can survive in a wide range of temperature from -1.8°C to 113°C .
- Hence archaea and their proteins are of commercial interest as they live and grow in unusual physiochemical conditions.
- Archaeal proteins which are halophilic or especially resistant to extreme pH values are also important

Haloarchaea and their Retinal Proteins

- All the Six genera of halophilic archaea that have been identified tend to produce retinal proteins.
- Sensory function is very common in eukaryotes other than that bacteria have a presumed energy conversion system due to the proteins.
- The light-driven proton pump bacteriorhodopsin drives a proton circuit across the cell membrane,
- ATP is produced via photophosphorylation as the chemical energy source for cell growth.

Haloarchaea and their Retinal Proteins



Halobacterium salinarum is found in nature in concentrated salt solutions as they occur in salines.

A purple color is caused by bacteriorhodopsin, and this is the key protein of the photosynthetic capabilities of *H. salinarum*. The proton pathway with the amino acids involved and the lysine-bound retinylidene residue are shown in the structural model of bacteriorhodopsin (foreground).

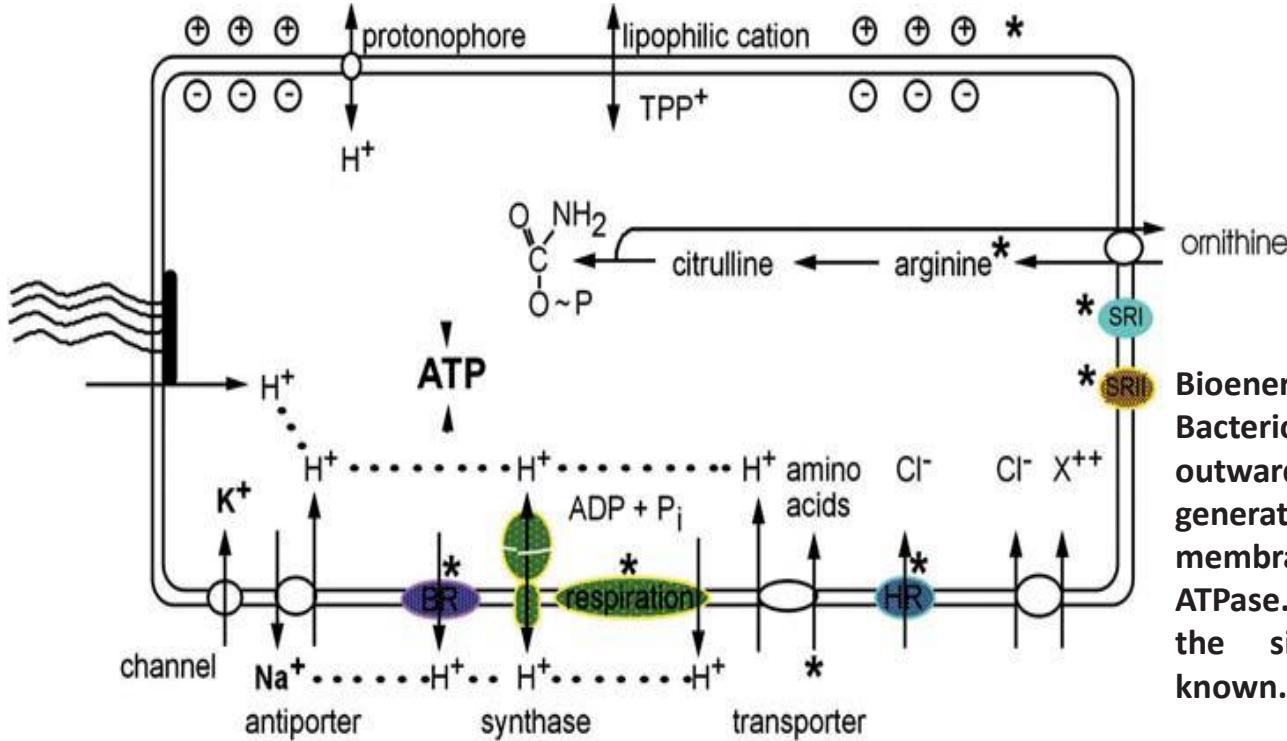
Haloarchaea and their Retinal Proteins

- Halophilic archaea, among them the genus Halobacteria, are unique in the sense that they are the only group of archaea that contains retinal proteins.
- Two principal functions of retinal proteins are sensory function (eukaryotes) or a presumed energy-converting function (bacteria).
- Only halophilic archaea have developed a set of four retinal proteins, two of which serve a sensory function, while two convert light energy to chemical energy.

Haloarchaea and their Retinal Proteins

- Retinal, or vitamin-A aldehyde, originates from β -carotene by oxidative cleavage in the centre of the molecule.
- The aldehyde in the free state is a chemically labile molecule with five conjugated double bonds.
- It is oxygen-sensitive and shows light-induced isomerization around all double bonds.
- Light and oxygen together (photooxidation) destroy the free retinal easily.
- All known proteins containing retinal, protect the molecule against photooxidation and select specific photoisomerization reactions
- 11-*cis* retinal to all-*trans* retinal (in visual pigment)
- all-*trans* retinal to 13-*cis* retinal (in haloarchaeal proteins)
- All retinal proteins are intrinsic membrane proteins and posses trans membrane topography
- Retial always binds to ϵ - amino group of a lysine of seventh transmembrane helix (protonated schiff base)

Haloarchaea and their Retinal Proteins



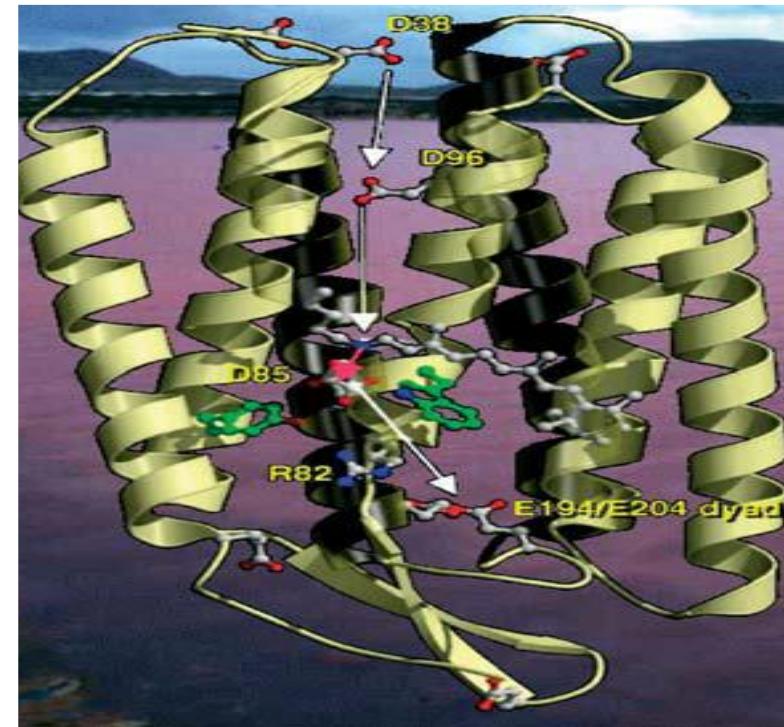
Bioenergetics of *Halobacterium salinarum*. Bacteriorhodopsin acts as a light-driven, outward directed proton pump. The generated proton gradient over the cell membrane drives a membrane-bound ATPase. These two proteins together form the simplest photosynthetic system known.

Structure and Function of

- Bacteriorhodopsin is an intrinsic membrane protein with the common seven-transmembrane helix topology and an approximate molecular weight of 26 kDa.
- The seven helices are arranged in two arcs :
 - a) An inner arc with helices B, C, and D.
 - b) An outer arc with helices E, F, G, and A.
- A transmembrane pore is formed mainly between helices B, C, F, and G.

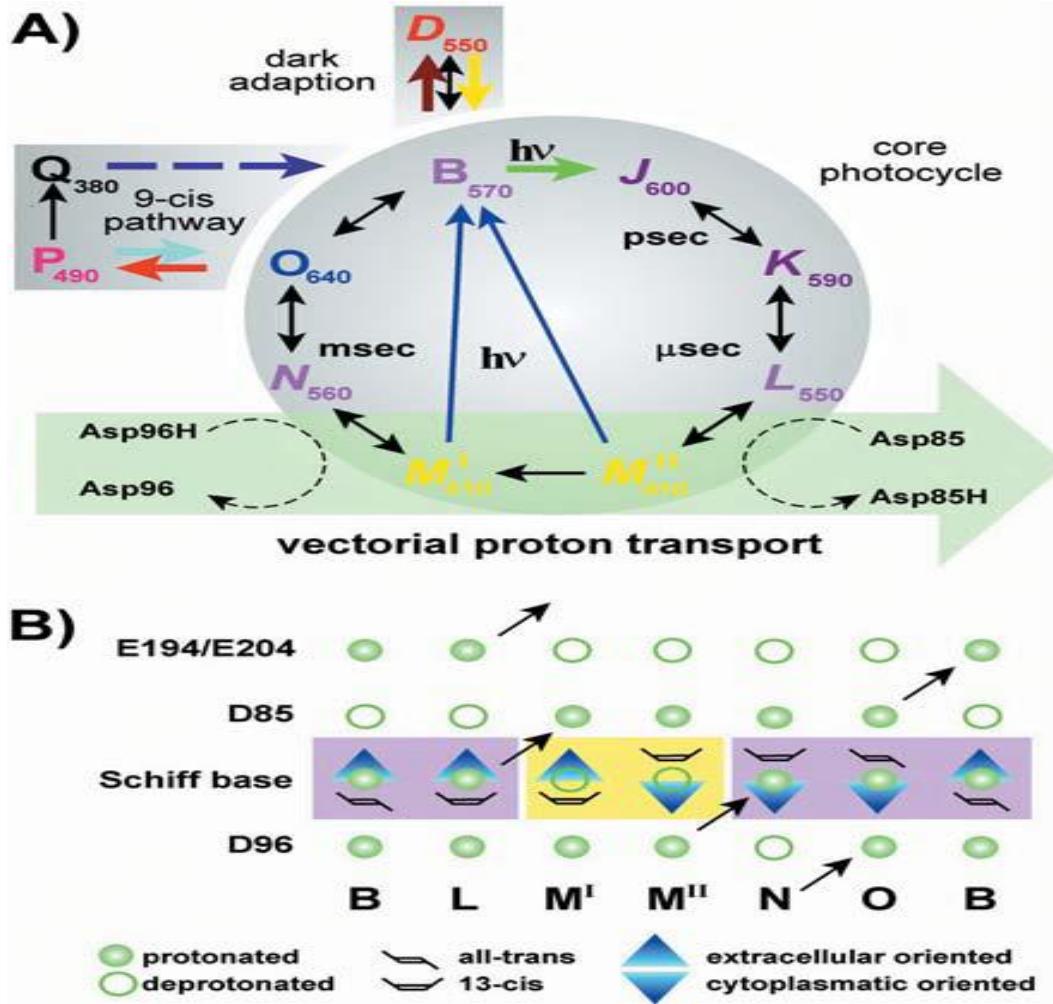
Structure and Function of Bacteriorhodopsin

- The retinal is bound to Lys216 in helix G as a protonated Schiff base, which interrupts the pore and separates an extracellular (EC) half channel from a cytoplasmic (CP) half channel.



Structure and Function of Bacteriorhodopsin

- Light absorption causes photoisomerization of the retinal (all-trans to 13-cis and vice versa).
- This cycle may formally be represented as a sequence of six steps which are indispensable for transport :
 - a) All-trans to 13-cis photoisomerization and its thermal reversal (isomerization, I).
 - b) Reversible change in accessibility (switch, S) of the Schiff base for ions in the EC and CP channel respectively.
 - c) and ion transfer (T) reactions to and from the Schiff base active center.



Photocycle of bacteriorhodopsin.

- (A) Upon absorption of a photon, the initial B-state of bacteriorhodopsin is converted photochemically to the J-state from where a series of thermal steps leads back to the initial state. The proton transport is intimately coupled to the photocycle, which is observed as a sequence of intermediates which are represented by the common single-letter code with their absorption maxima given as subscripts. In the dark, bacteriorhodopsin relaxes thermally to the D-state which has 13-*cis* configuration. The resulting mixture of B- and D-states is called dark adapted bacteriorhodopsin. From the O-state, a photochemical conversion of all-*trans* to 9-*cis* retinal is possible which is not thermally reisomerized to the initial state.
- (B) The proton transport and related retinal configurations as well as accessibility of the nitrogen in the Schiff-base linkage between retinal and Lys216 are indicated. This sequence represents several of the molecular changes involved in the proton transport.

Applications

- Light driven anion pump transports Cl^- ions against salt (proton pump) to maintain cellular potential to prevent exosmosis.
- Based on these there are some industrial applications
- Because of these proton pumps, proton motive force can be generated and that can be converted to charge.
- The three most important features which eventually lead to biotechnological applications of bacteriorhodopsin on the basis of its catalytic cycle are:

Applications(Exploited Functions)

1. The color changes, which can be used for any type of information processing and storage process.
2. The photoelectric events which are due to the changing geometry of the Schiff base upon photoisomerization and the movement of the proton. Such electric changes occur from the picosecond to the millisecond time regime.
3. The pH change between the inside and the outside of bacteriorhodopsin-containing membrane systems as the net result of proton translocation.
4. 3D array sensor functions.

Applications (overview)

- So far, the color changes are under most intensive investigation because of the velocity of light-triggered reactions in bacteriorhodopsin and the possibility of regulating the speed of the color changes over a very wide range.
- **“Artificial Retina”**- Sensor Array.
- Genetic modifications to produce bacteriorhodopsin mutants.
- Purple Membranes- 2D crystals of Bacteriorhodopsin *in vivo*. A source of biomaterial in information technology.

Bacteriorhodopsin (Part II)

Its Potential in
Technical
Applications

Function of BR

- The 3 most important features that lead to use of BR based on catalytic cycle :
- 1) The colour change (for information processing and storage process)
- 2) The photoelectric event due to changing geometry of schiff base upon photoisomerization and movement of proton (ps to ms time regime)
- 3) pH change between inside and outside of BR membrane system.
- Four molecular stucture of retinal proteins are presently known 1. Rhodopsin 2. Bacteriorhodopsin 3. Halorhodopsin 4. Sensory rhodopsin
- Sensory Rhodopsin I & II :
 - Receives orange light- attractive stimulant
 - Receives blue light or near UV – Repulsive Stimulant. (regulates flagellar motor)

Biotechnological Production of BR

Simple to isolate, chemical and photochemical stability when in the form of purple membrane

BR forms 2D crystal in vivo and can be isolated as purple membranes

Isolation is facilitated by two facts:

1. Halobacterial cells are unstable in water and cell constituents are released upon lysis
2. The cell membrane is fragmented and crystalline patches of purple membrane are set free as fragments of largest size and highest buoyant density

Hence can be purified by centrifugation or filtration (95-100% pure)

Current production of BR is 25 g m^{-3} nutrient growth

Technical applications of BR

In most of the applications BR is used in the purple membrane
Three basic molecular functions which may be used for technical
applications:

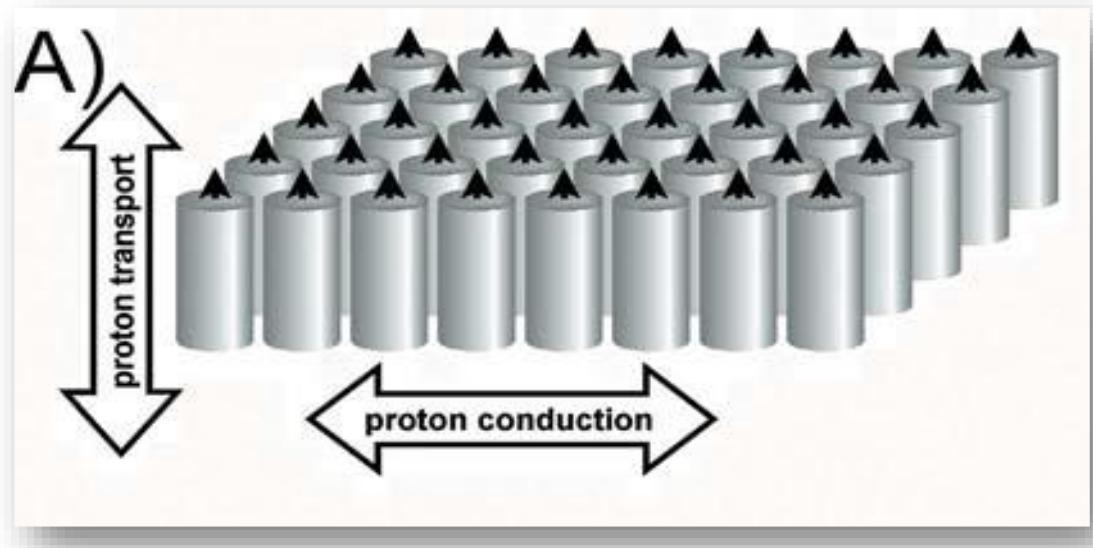
1. Photoelectric
2. Photochromic
3. Proton transport properties

Photoelectric Applications

- The use of bacteriorhodopsin as a molecular level photoelectric conversion element is one of the fields where technical applications of the material have been examined.
- A photovoltage up to 250 mV per single bacteriorhodopsin layer is generated.
- Can be used either as an indicator or control element for various applications such as:
- Sensor, Capacitor, 3D Data storage material, Molecular Devices (Spatial Light Modulators), Artificial Retina.

Photoelectric Applications

- Triggered by the absorption of a photon, the bacteriorhodopsin molecule undergoes a series of very rapid molecular changes.
- one of which is the generation of a photovoltage caused by changes in the orientation of molecular dipole moments that are triggered by the isomerization of the retinal.
- The proton released through the outer proton half channel may be either transferred to the outer medium or conducted along the surface of the PM.
- A single PM sheet contains several thousands of unidirectionally oriented BR molecules.



(A) Absorption of a photon by bacteriorhodopsin causes molecular changes which lead on a pico- to nanosecond timescale to the generation of a photovoltage over the molecule. The bacteriorhodopsin molecules in a single purple membrane (PM) patch are unidirectionally oriented. The light-driven vectorial proton transport of all the bacteriorhodopsin molecules is switched in parallel. This means that, over a single PM patch, it is not the voltage but the proton current which is proportional to the light intensity. The protons transported through the bacteriorhodopsin molecules can either be released to the outer medium or move along the surface of the PM patch due to proton conduction.

Photoelectric Applications

- Upon illumination, a number of bacteriorhodopsin molecules proportional to the intensity of light will be excited to accomplish a proton transport process.
- All of the bacteriorhodopsin molecules in a single PM patch are oriented in the same direction
- The voltage generated over a single membrane is independent of the number of active molecules
- But the proton current generated is proportional to the light intensity.

Photoelectric Applications

- A voltage is induced only during the light intensity change.
- The polarity of the signal is different for the OFF → ON and the
- ON → OFF transition, called the “**differential responsivity**” of PM layers.

Preparation of Oriented PM Layers

- The photoelectric signal of PM is high enough so that a single layer only of PM is needed for applications.
- The PM acts as a photoelectric indicator molecule.
- The Langmuir-Blodgett technique is often used for the preparation of such devices

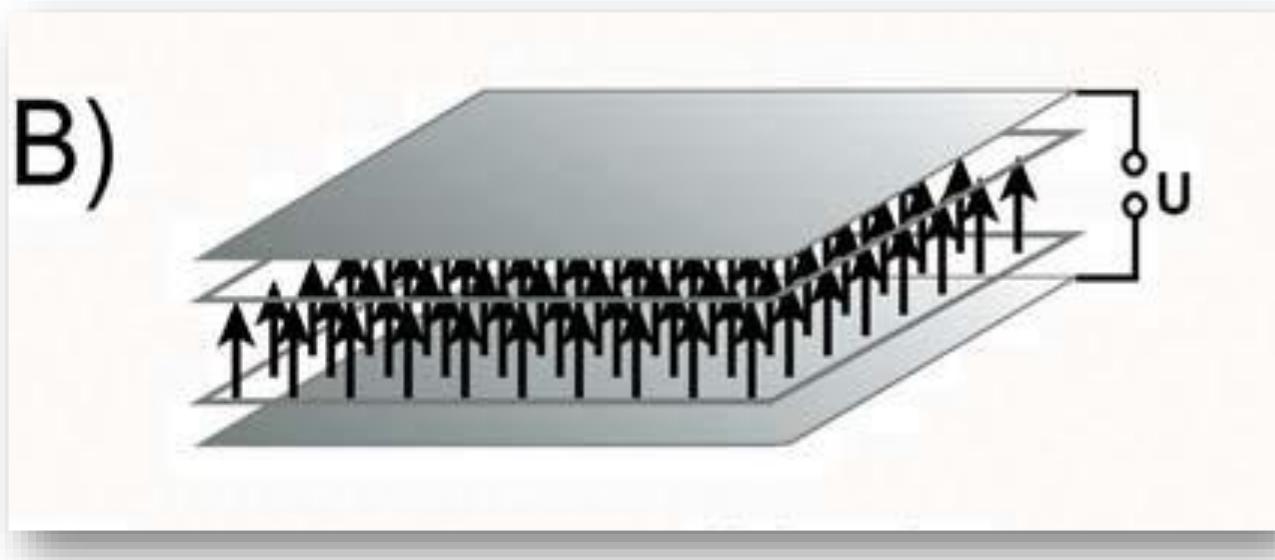
Photoelectric Applications

- By covalent crosslinking or by polymer embedding of the PM patches to further fix the layer.
- By coating the surface with monoclonal antibodies generated against one side of the plasma membrane .
- The antibodies selectively react with the cytoplasmic or extracellular side of the PM. Thus, a highly oriented monolayer of PM can be obtained using this method.

Photoelectric Applications

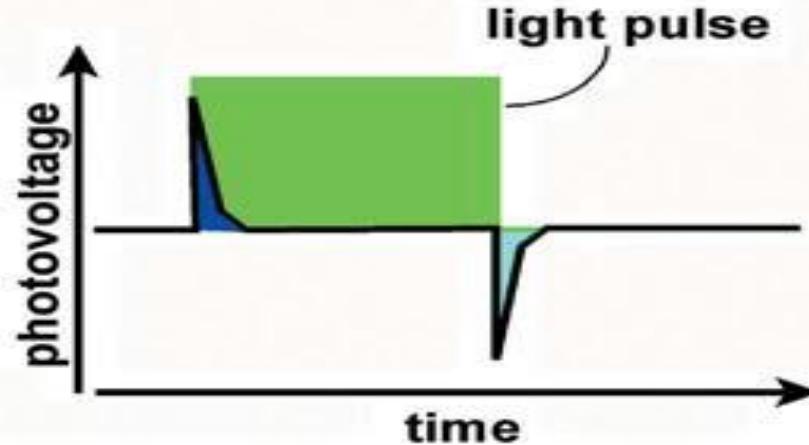
Interfacing the Proton-Motive Force.

- Interfacing the proton-motive force of BR with the electron-conducting outer electrodes requires an electrolyte layer which couples both “worlds”.

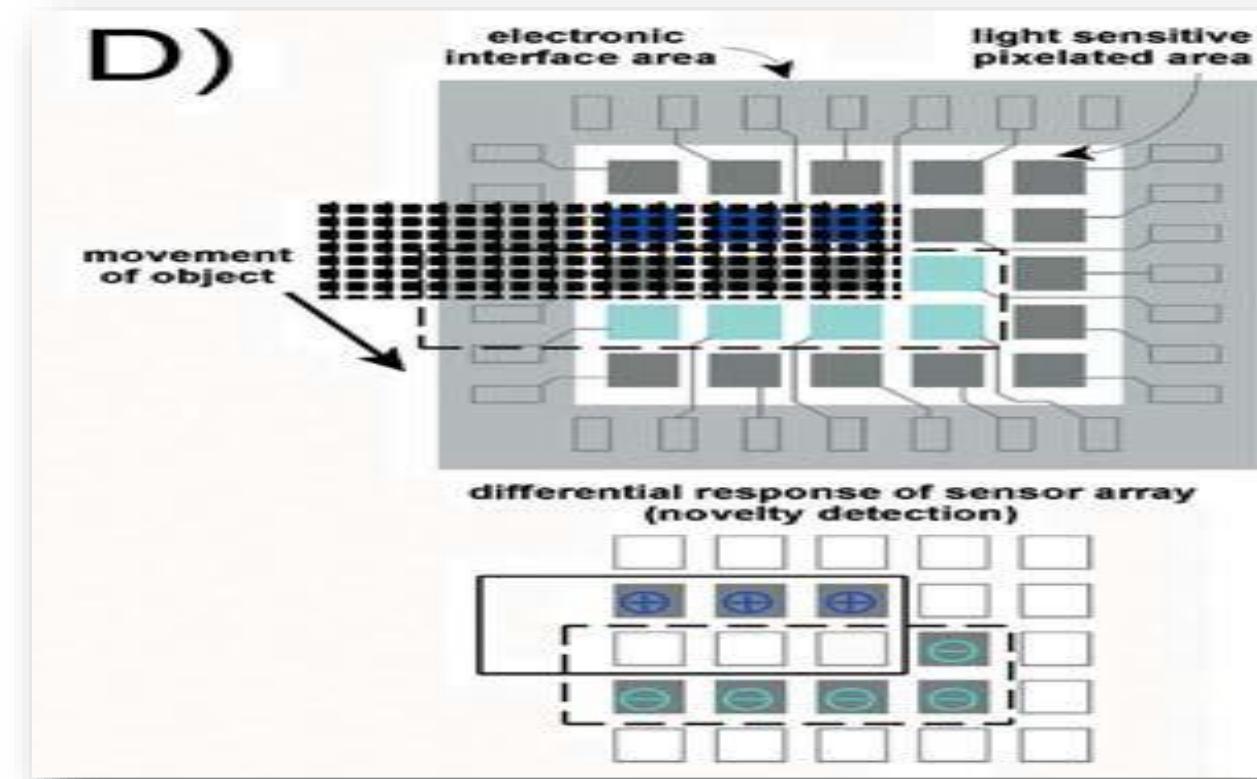


(B) Oriented bacteriorhodopsin molecules, each represented by an arrow, sandwiched in a capacitor structure can be used as a photoelectric indicator cell. Upon illumination, a charge separation over the bacteriorhodopsin layer is generated which induces a proportional charging of the outer electrode layers. No electric conduction between the bacteriorhodopsin layer and the electrodes is required. The electric field of the charge distribution induced in the electrodes compensates the electric field caused by bacteriorhodopsin.

C)



(C) Depending on the type of outer circuitry, either the induced voltage or the induced charge motion can be measured. In the latter case, a signal is recorded which corresponds to the first derivative of the temporal change of the light.



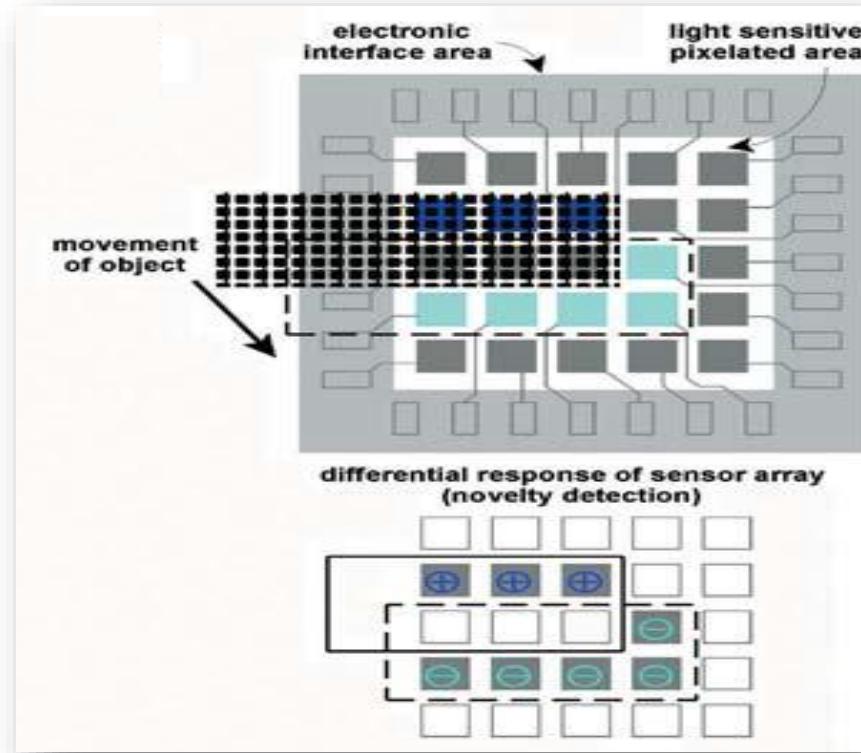
(D) In a pixelated structure which is coated with oriented bacteriorhodopsin, photovoltages are measured only in those spots where a change in the light intensity occurs; hence, this is called novelty filtering.

Major Applications

- Ultrafast photodetection
- 2D Photoelectric arrays
- Artificial Retinas
- Control elements for a liquid crystal spatial light modulator.
- Indicator of 3D memories.

Artificial Retinas

- Edge detection
- Assume the dark rectangular structure shown in Figure prevents a set of electrodes from being exposed to light.
- If this structure is moved over the light-sensitive sensor area (see arrow), it causes a voltage to be induced in each of the pixel electrodes, with a sign proportional to the light change.
- Because only those electrodes “fire” where a change of the illumination occurs.
- This type of sensor is called an “artificial retina”, and this type of preprocessing is called “novelty filtering”.



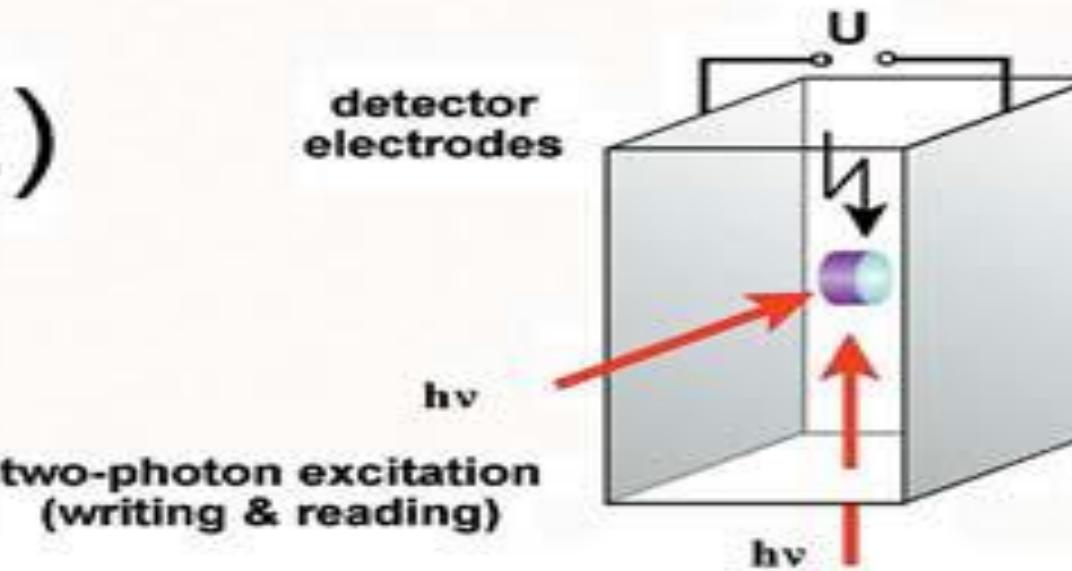
Electro-optically controlled spatial light modulators

- The amplifier electronics which detects the photovoltages induced in the electrode pixels may be connected to a SLM device.
- Liquid crystal (LC) -based SLMs are state-of-the-art.
- The electrodes of the bacteriorhodopsin-based artificial retina are connected one-to-one to the pixels of a LC-SLM
- The LC-layer of the SLM is controlled directly by the artificial retina device.

Readout in 3-D Memories

- Readout of volume storage units with bacteriorhodopsin.
- The basis is a cube of oriented PM patches in either the purple initial state or the yellow M state.
- Upon illumination of a PM patch in the initial state (which may be addressed by actinic light), a photovoltage signal is induced.
- A PM patch in the M state would not respond to the actinic light
- The two electrodes on the outer surfaces of the bacteriorhodopsin cube would detect the photovoltage generated.
- By this method the 3-D distribution of the photochemical states of PM patches could be read out

E)



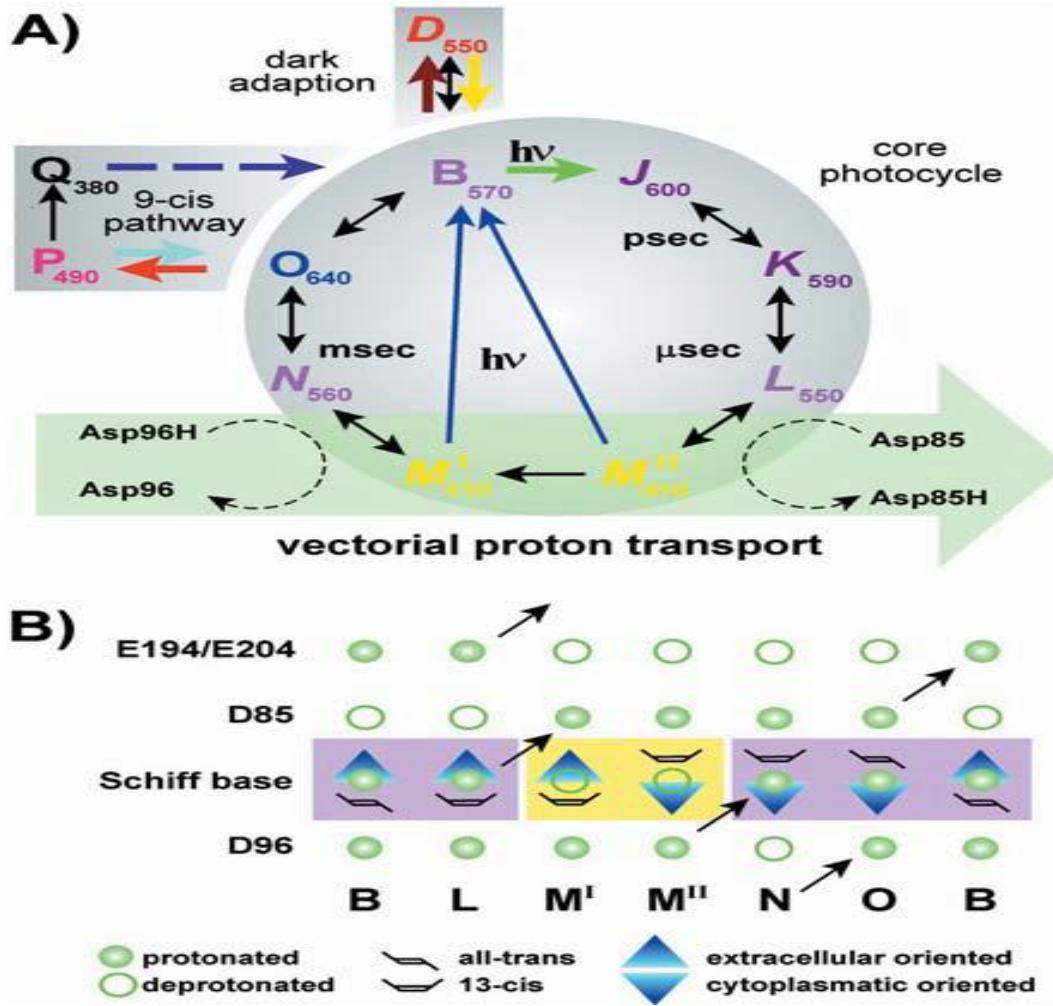
(E) In a volume (e. g., in 3-D data storage) the detection of the photovoltage in an outer capacitor structure was considered for readout. If the bacteriorhodopsin in the point of excitation is in the B-state, the absorption of a photon will lead to a photo-induced voltage, but not in the M-state.

Bacteriorhodopsin (Part III)

Its Potential in
Technical
Applications

Photochromic Applications

- Isomerization from all-*trans* to 13-*cis* is the first occurrence after the photochemical excitation of bacteriorhodopsin.
- This causes significant transient shifts in the absorption spectrum.
- Change in isomerization and deprotonation of the chromophoric group is observed.
- In the L to M transition, deprotonation occurs from the Schiff base nitrogen group to Asp85 causing a drastic blue shift of the absorption to 410 nm.



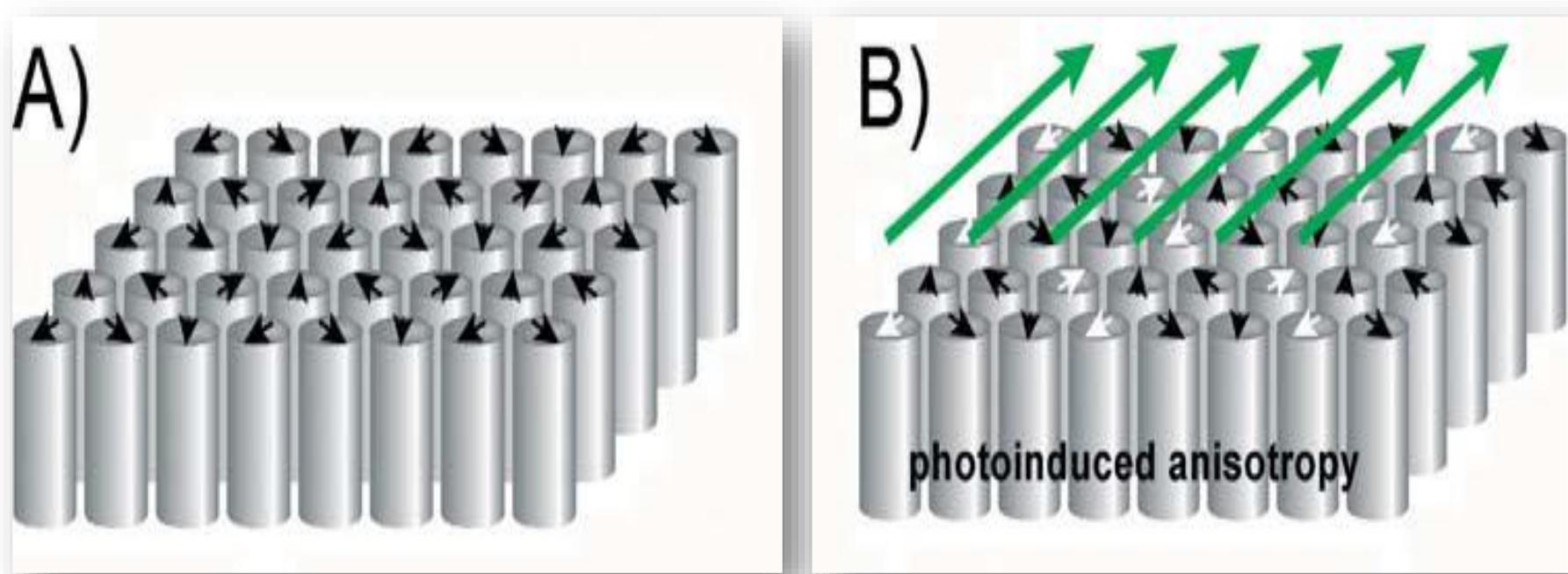
Photocycle of bacteriorhodopsin.

(A) Upon absorption of a photon, the initial B-state of bacteriorhodopsin is converted photochemically to the J-state from where a series of thermal steps leads back to the initial state. The proton transport is intimately coupled to the photocycle, which is observed as a sequence of intermediates which are represented by the common single-letter code with their absorption maxima given as subscripts. In the dark, bacteriorhodopsin relaxes thermally to the D-state which has 13-*cis* configuration. The resulting mixture of B- and D-states is called dark adapted bacteriorhodopsin. From the O-state, a photochemical conversion of all-*trans* to 9-*cis* retinal is possible which is not thermally reisomerized to the initial state.

(B) The proton transport and related retinal configurations as well as accessibility of the nitrogen in the Schiff-base linkage between retinal and Lys216 are indicated. This sequence represents several of the molecular changes involved in the proton transport.

Photochromic Applications

- The photochromism of bacteriorhodopsin is dominated by the intermediate which has the longest life-time.
- This forms a “**bottleneck**” in the photocycle.
- The anisotropy of the retinylidene groups causes excitation of a random distribution of PM patches with polarized light (Figure A).
- Further, causing the chromophores to become oriented in parallel to the actinic light polarization and a preferentially converted anisotropy is obtained (Figure B).

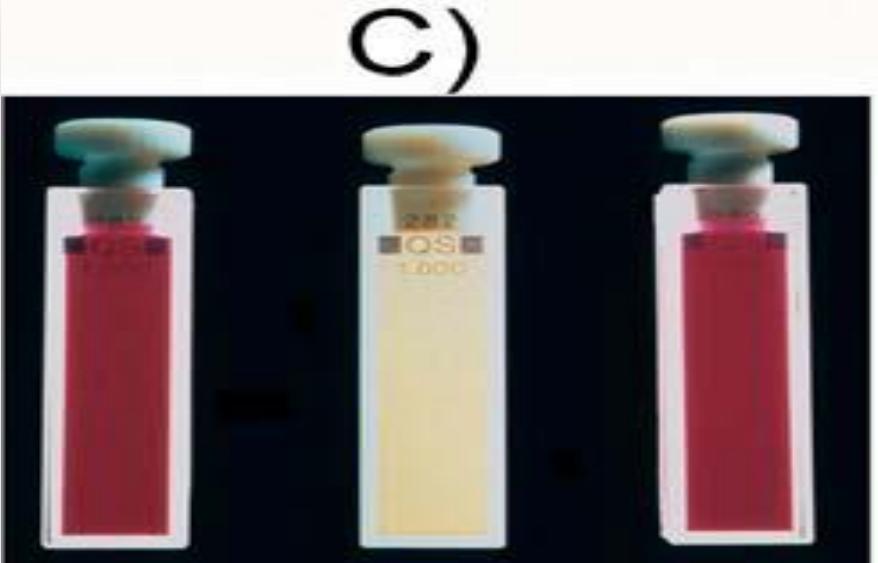


(A) The retinylidene residues are strongly anisotropic.

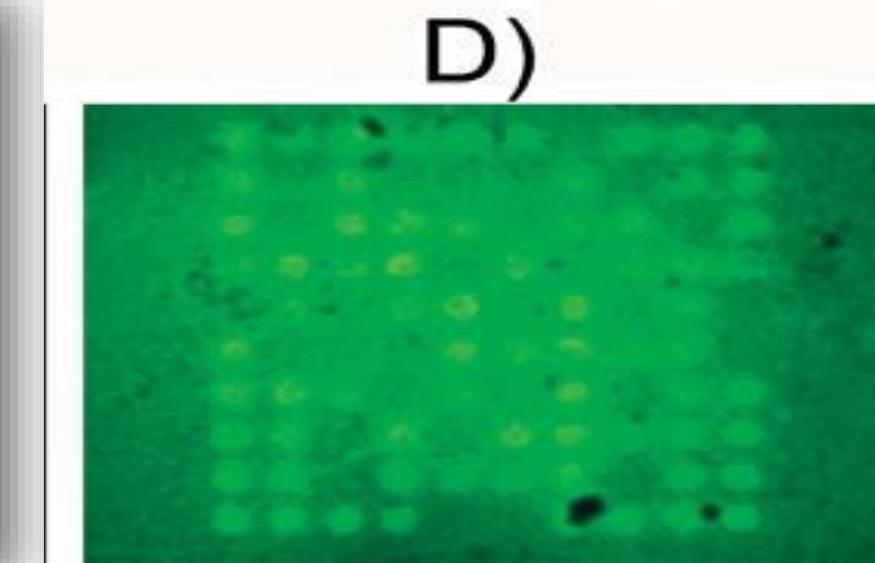
(B) Upon illumination with polarized light, the retinylidene residues which are in parallel to the electric field vector of the actinic light are preferentially excited and isomerized.

Photochromic Applications

- In solutions containing PM this is masked by diffusion.
- In bacteriorhodopsin-films where the PM patches are fixed, the photoinduced anisotropy can be easily observed and utilized.
- Three types of photochromic changes in bacteriorhodopsin :
 - 1) The photochromic shift between the B and M states - Optical processing tasks (Figure C).
 - 2) 9-cis-containing states of blue membrane or suitably modified BR-variants - Photoerasable data storage (Figure D).



(C) Transient photochromic change of bacteriorhodopsin between the initial purple state and the yellowish M-state (middle).



(D) The photochemical formation of *9-cis* retinal may be utilized for photochromic long-term storage.

Photochromic Applications

3) Permanent photochromic changes obtained through two-photon absorption in bacteriorhodopsin - Long-term data storage (Figure E)



**Permanent storage of information
in bacteriorhodopsin.**

Preparation of Bacteriorhodopsin Films

- Optical films are prepared from bacteriorhodopsin by polymer embedding.
- Optically clear and water-soluble polymers are suitable (e. g., polyvinylalcohol, gelatin)
- The film formation is usually carried out by mixing the polymers with PMs and additives in aqueous solution casted on a glass support.
- **Usually dried** in air to remove water.
- The films may also be sealed with a second glass plate.

Interfacing the Photochromic Changes

- Ease of implementation of the interface between the bacteriorhodopsin films and any type of optical system.
- The bacteriorhodopsin film is completely sealed, the only interface being the light which transports energy and information simultaneously.

Applications :

- **Photochromic colour classifier:** the use of bacteriorhodopsins with different absorption maxima would allow a biomimetic system for color perception to be set up.

Interfacing the Photochromic Changes

- **Photochromic inks:** These inks differ from the polymer films for optical recording by their rheological properties. Depending on the method of application (e. g., screen printing, offset printing), the viscosity and surface tension must be considered.
- **Electrochromic inks :** The main color shift in bacteriorhodopsin is due not to a primary photochemical reaction but to the protonation change of specific groups. As protons are charged particles, their removal from the binding position by electric fields is possible.

Interfacing the Photochromic Changes

- **Photochromic inks:**
- **Examples** of photochromic inks made from bacteriorhodopsin are shown in Fig C, with the initial colored (purple) and bleached, yellowish inks in the foreground and background, respectively.



Interfacing the Photochromic Changes

- **Photochromic photographic film:** Permanent bleaching of bacteriorhodopsin may be achieved with hydroxylamine.
- The chemical reaction of hydroxylamine with the retinal binding site occurs in an intermediate state only.
- No reaction of BR in the B-state is observed. With **this**, it is possible to fabricate nonreversible optical films from bacteriorhodopsin.
- **Long-term photorewritable storage of information :** In blue membrane, a photochemical conversion from *all-trans* to *9-cis* retinal, which appears pink in bacteriorhodopsin, may be induced by high light intensities.

Interfacing the Photochromic Changes

- **Neural networks :** Bacteriorhodopsin is also a suitable material for neural networks because its absorption state may be shifted with blue and yellow light in different directions.
- **3-D information storage :** Bacteriorhodopsin shows an astonishingly high two-photon absorption cross-section of the initial B state.
- Can be used in a two-photon absorption set-up to address the absorption state of bacteriorhodopsin in three dimensions.
- Advantage of such a memory device is its tolerance towards electromagnetic radiation.

Interfacing the Photochromic Changes

- **3-D information storage :**

- An ID card sample with a bacteriorhodopsin-based optical storage in the purple-colored strip is shown in Fig D

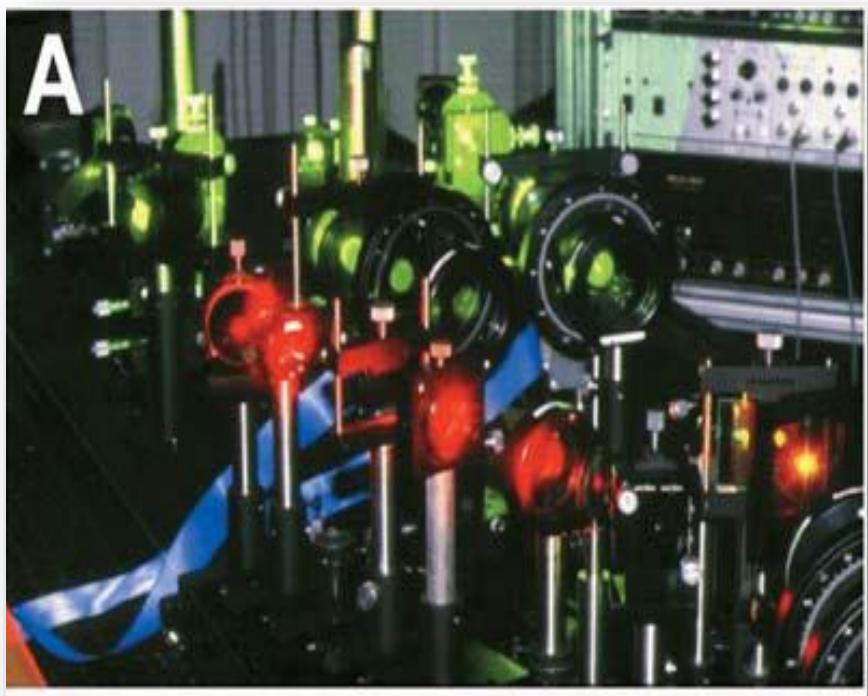


Interfacing the Photochromic Changes

- **Nonlinear optical filtering:** the nonlinear optical response of bacteriorhodopsin towards the incident light intensity
 - image processing purposes (e. g., edge enhancement, noise reduction)
 - The response curve of the bacteriorhodopsin can be tuned over several orders of magnitude by changing the lifetime of the M-state.
 - **Can be** accomplished by: changing the pH, or by using modified bacteriorhodopsins.

Interfacing the Photochromic Changes

- **Holographic pattern recognition and interferometry:** applications include
 - a holographic real-time correlator (Fig A)
 - a holographic camera for nondestructive testing (Fig B).

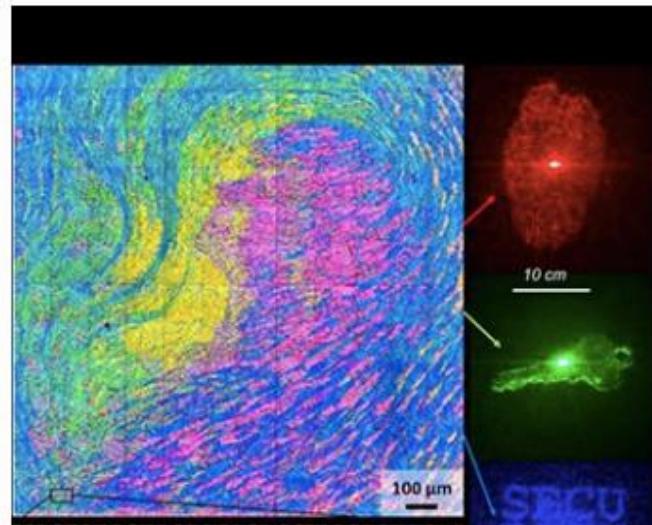


Photochromic applications of bacteriorhodopsin. (A) Holographic correlator; (B) holographic camera for interferometric testing;

Holographic pattern

Sarah Michaud

Researchers from the Singapore University of Technology and Design (SUTD) have demonstrated a method for embedding multiple holograms into a color print using nanostructured pixels that can control the phase and amplitude of light (Nat. Commun., doi: [10.1038/s41467-018-07808-4](https://doi.org/10.1038/s41467-018-07808-4)). Under ambient light, the holographic color prints appear as a color image. However, when the prints are exposed to red, green or blue light from a hand-held laser pointer, they project

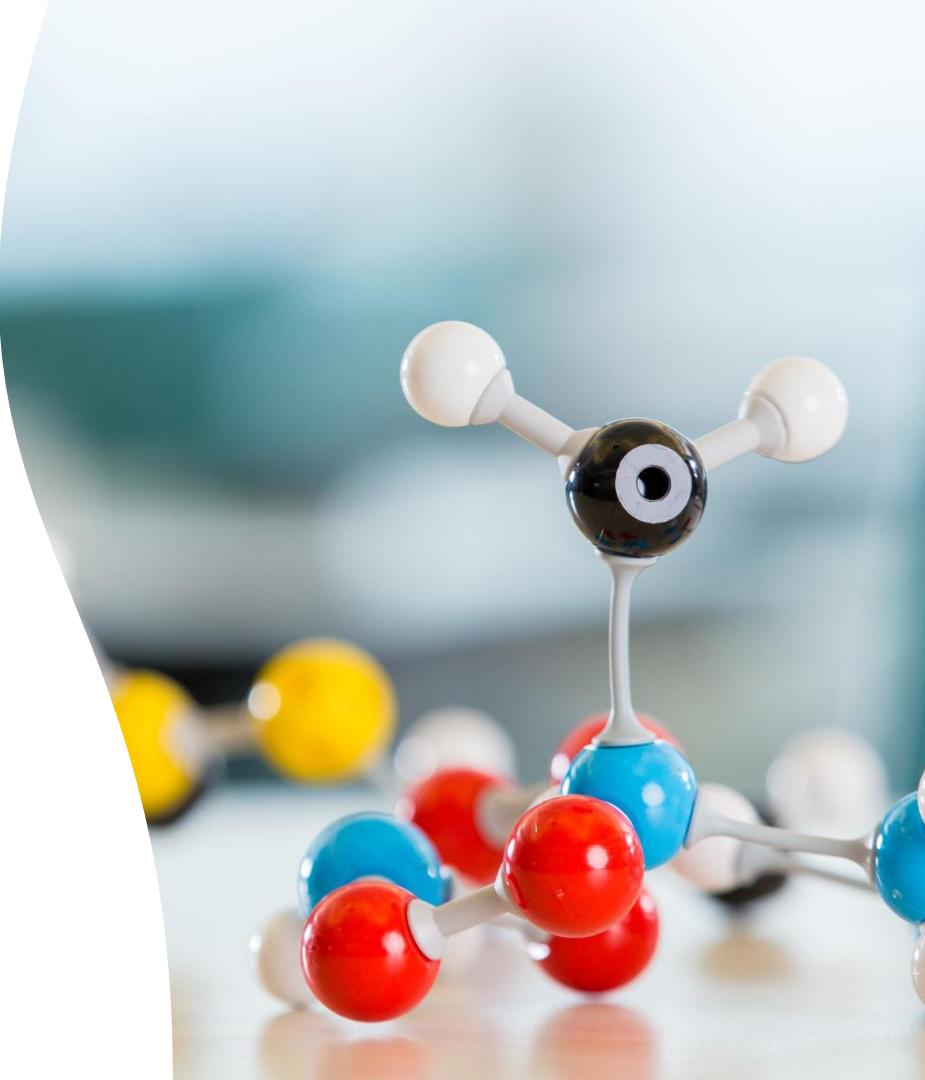


Outlook

- Bacteriorhodopsin is today the biological photochromic material for which technical applications in optical information processing are much more developed than for any other biomaterial.
- Biomaterials as blueprints for technical materials with nanoscale functions form the basis of the concept of nano bionics and bacteriorhodopsin was the first such example.

Bionanotechnology
BBL 747

Molecular Imprinting

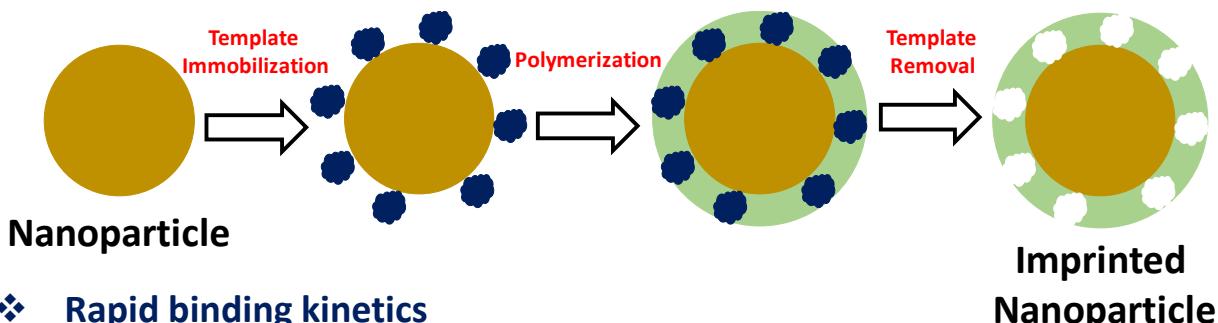
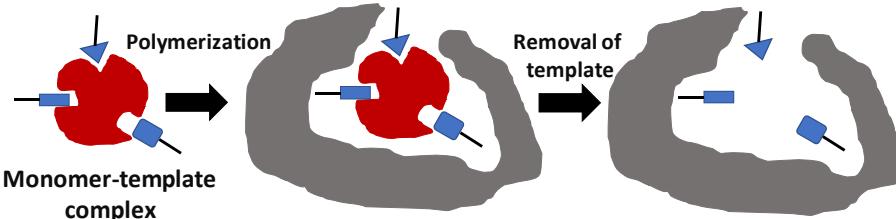


Introduction

- Molecular imprinting is defined as “**the construction of ligand selective recognition sites in synthetic polymers**
- where a template (atom, ion, molecule, complex or a molecular, ionic or macromolecular assembly, including micro-organisms) is employed in order to facilitate recognition site formation during the covalent assembly of the bulk phase by a polymerization or polycondensation process,
- with subsequent removal of some or all of the template being necessary for recognition to occur in the spaces vacated by the templating species.”
- The **first MI-based synthetic receptors reported by Wulff in 1972**
- Compared to other recognition systems, MIPs, which possess three major unique features **of structure predictability, recognition specificity and application universality.**
- Application in many fields, such as purification and separation, chemo/biosensing, artificial antibodies, drug delivery, and catalysis and degradation, owing to their high physical stability, straightforward preparation, remarkable robustness and low cost

Surface Molecular Imprinting to Develop Biorecognition Elements

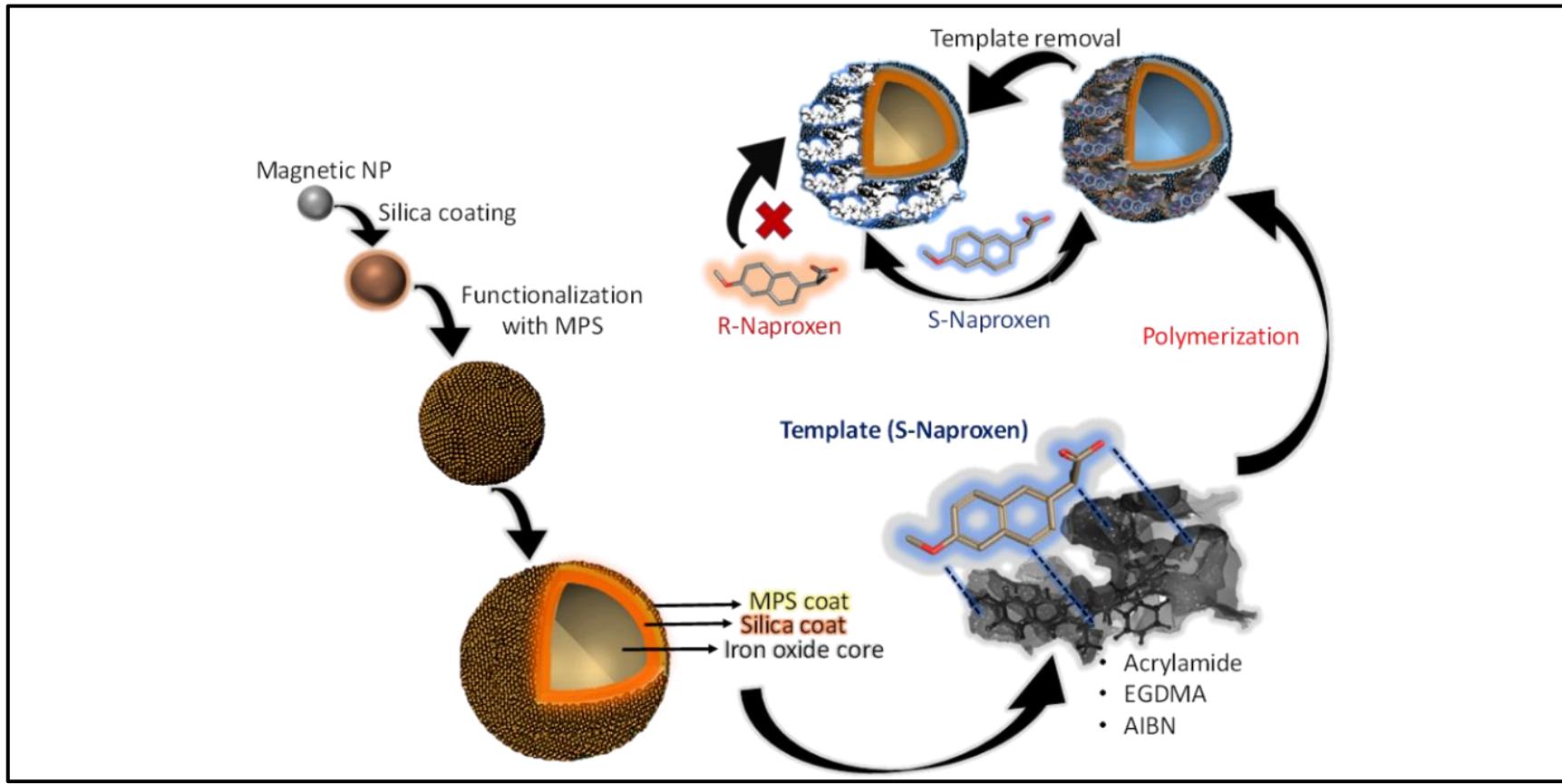
- ❑ Classical approach:
incorporate template and
monomers all together
- ❑ Difficulty in removing template



- ❖ Rapid binding kinetics
- ❖ Traces of template being easily removed
- ❖ More flexible incorporation of multifunctionality
- ❖ High surface-to-volume ratio, High level of specific binding sites
- ❖ Better control of manufacturing process



**“Lock-key”
Molecular lock**



Schematic representation for the synthesis of molecularly imprinted magnetic nanoparticles for S-Naproxen and the selective binding with the template (**Goyal et al ACS Applied Nanomaterials 2019**) (3-Mercaptopropyl)trimethoxysilane (**MPS**)

Fundamentals of MIPs

The process of molecular imprinting involves:

- **the polymerization of a functional monomer and**
- **a cross-linker around a molecular template.**
- Firstly, template–monomer complexes are achieved between a template molecule and a complementary functional monomer, the exact constellation of which distinguishes the different types of molecular imprinting technologies from each other
- A crosslinking polymerization reaction is then performed around the complex.
- After the template molecule is extracted, the imprinted sites contain **a three-dimensional network presenting pores** with the geometry and position of the functional groups complementary to those of the templates
- Two main **methods to produce MIPs, i.e., based on covalent and noncovalent interactions between the template and the functional monomer.**
- Covalent imprinting, being stoichiometric, ensures that functional monomer residues exist only in the imprinted cavities.

Fundamentals of MIPs

- Covalent imprinting often uses readily reversible condensation reactions involving boronate esters, ketals/acetals, and Schiff's base.
- However, covalent imprinting is regarded as a less flexible method since the reversible condensation reactions are limited.
- To reach thermodynamic equilibrium is very difficult since the strong covalent interactions result in slow binding and dissociation.
- Noncovalent imprinting can proceed by ionic interactions, hydrogen bonding, van der Waals forces and $\pi-\pi$ interactions.
- Most common, the dominant interaction is hydrogen bonding

Fundamentals of MIPs

- Recently, noncovalent imprinting has become the most popular and general synthesis strategy due to the **simplicity of operation and rapidity of binding and removal**.
- However, **noncovalent imprinting is sensitive to even slight disruption of the interactions holding the complex together** (for example, the presence of water), and it is therefore not very robust.
- In order to combine the durability of covalent imprinting and the rapid target uptake of noncovalent imprinting, a new method called **semi-covalent imprinting** has emerged.
- This method offers an intermediate alternative in which **the template is bound covalently to the functional monomer, but template rebinding is based on noncovalent interactions**.

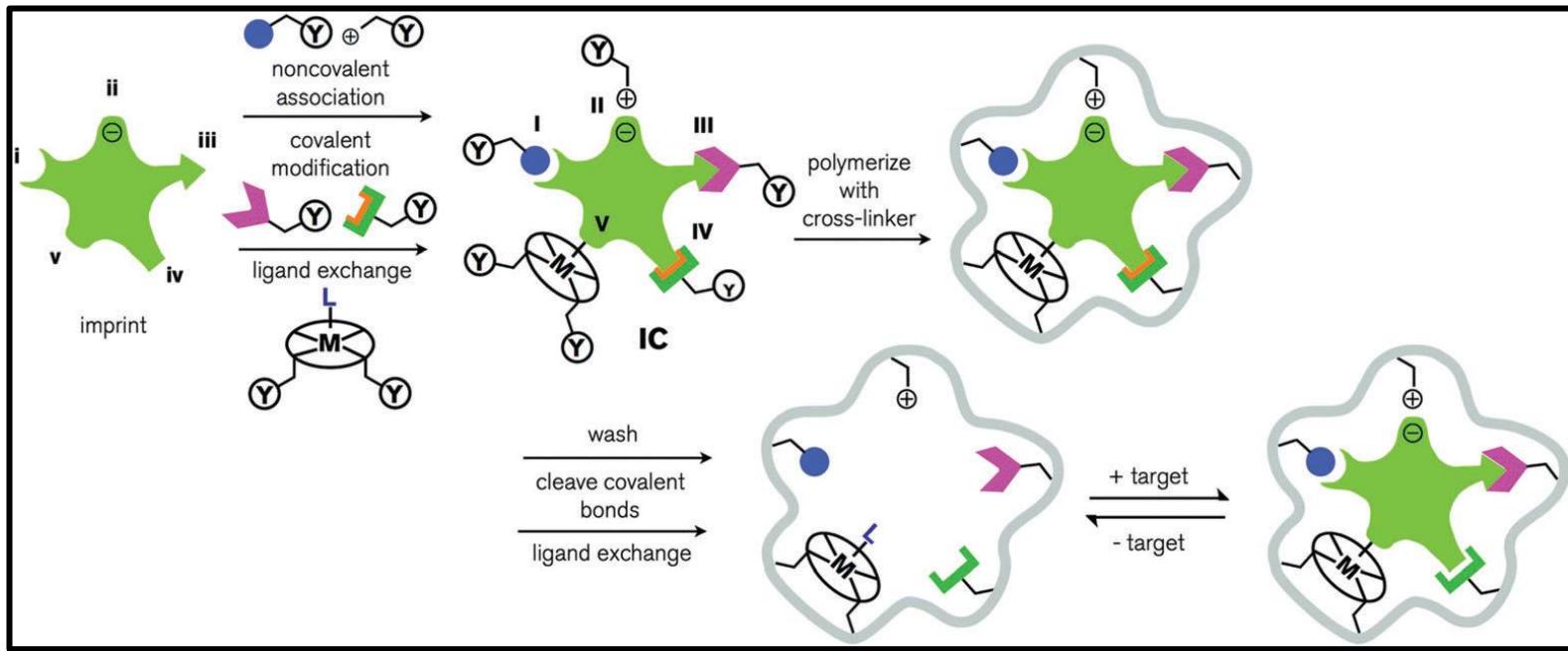


Fig. Five main types of molecular imprinting: (i) noncovalent, (ii) electrostatic/ionic, (iii) covalent, (iv) semi-covalent, and (v) metal centre coordination. An imprint molecule is combined with an appropriately chosen functional monomer, through noncovalent, covalent, or ligand (L) to metal (M) interactions with complementary functional groups on the imprint. A complex of the imprint and functional monomer (IC) is formed, in which the functional monomer is bound to the imprint molecule (I) by hydrogen bonding or van der Waals interactions, (II) by electrostatic or ionic interactions (the charges on the imprint and functional monomer may be reversed), (III) through a covalent bond, (IV) through a covalent bond with a spacer (orange), or (V) by ligand–metal or metal–ligand coordination. The functional monomer contains a functional group, Y, which undergoes a cross-linking reaction with an appropriate cross-linker. After polymerization of the complex with a cross-linker to form the solid polymer matrix (grey), the imprint functional monomer interactions are intact. The imprint is removed through washing, cleavage of chemical bonds, or ligand exchange, and leaves behind an imprint cavity with functional groups on the walls. Subsequent uptake of a target molecule is achieved by noncovalent interactions (in types i, ii and iv), the formation of a covalent bond (in type iii), or by ligand exchange (in type v) with target molecules that fit into the cavity and possess the correct structure. The matrix may also participate in target recognition and binding through non-specific surface interactions that result from surface features created around the imprint molecule during cross-linking.

Essential elements of molecular imprinting

- A typical MIP synthesis protocol contains a **template, a functional monomer, a cross-linker, a polymerization initiator and a solvent (porogen)**.
- In order to prepare MIPs with superior properties, numerous attempts have been made, since polymerization reaction is affected by many factors, such as **the type and amount of monomer, cross-linker, initiator and solvent and the temperature and time of polymerization reaction**.
- As we know, the “three-elements of molecular imprinting” include template molecules, functional monomers and cross-linkers, which should especially be investigated.

Target templates

- The ultimate goal of molecular imprinting is to generate MIPs with affinity and specificity comparable to those of the biological receptors so that they can eventually replace such biological entities in real applications.
- Generally, an ideal template molecule should satisfy three requirements:
 - it should **contain functional groups that do not prevent polymerization**;
 - it should exhibit **excellent chemical stability** during the polymerization reaction
 - it should contain **functional groups that can form complexes with functional monomers**.

Table 1 Common target templates used in molecular imprinting

| Type | Typical example |
|-------------------|---|
| Ions | Pb(II); Sr(II); Hg(II); CH ₃ Hg(I); Cd(II); Cu(II); Cr(III); Fe(III); Ni(II); UO ₂ ²⁺ ; Th(IV); Eu(III); As(III); PO ₄ ³⁻ |
| Organic molecules | Pesticides: atrazine; 2,4-dichlorophenoxyacetic acid; benzimidazole fungicides Endocrine disrupting chemicals: bisphenol A; estradiol; oestrone; polycyclic aromatic hydrocarbon (PAH) Explosive: 2,4,6,-trinitrotoluene (TNT) Pharmaceuticals: tetracycline; quinolones; propranolol; digoxin; sulfonamides Amino acids and peptides: tyrosine; alanine; tripeptides; helical peptides; cinchona alkaloids; N-terminal histidine sequence of dipeptides Sugars: D-fructose; D-glucose; D-galactose |
| Biomacromolecules | Lysozyme; adenosine; 3,5-cyclic monophosphate (cAMP); bovine serum albumin(BSA) |
| Cells and viruses | Tobacco mosaic virus; bovine leukemia virus; dengue virus; gut-homing T |

Functional monomers

- The role of the functional monomer is **to form a pre-polymerization complex with the template by providing functional groups.**
- So it is important to select a suitable functional monomer that can strongly interact with the template and form specific donor–receptor or antibody–antigen complexes prior to polymerization.
- Generally, a **functional monomer is comprised of two types of units- one is the recognition unit and the other is the polymerizable unit**
- MAA has been used as a “universal” functional monomer due to its hydrogen bond donor and acceptor characteristics.
- Dimerization of MAA modestly enhanced the imprinting effect
- high molar fractions of MAA would result in the large pore size of polymeric materials and further enhance the binding capacity of the polymers.

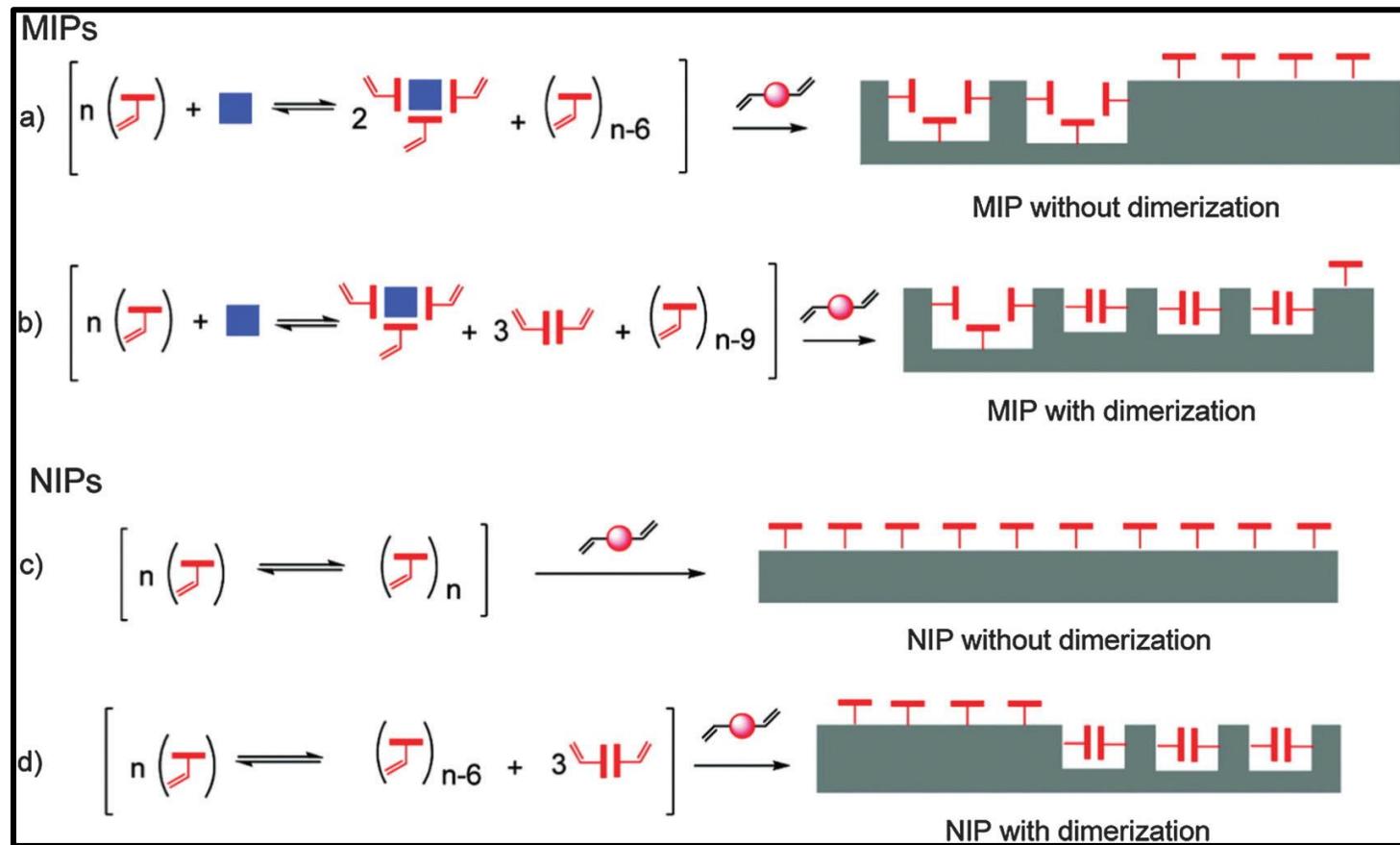


Fig. Illustration comparing imprinted (a and b) and nonimprinted polymers (c and d) formed from functional monomers that have or lack the ability to dimerize.

Molecular imprinting (Contd)Cross-linkers

- In the process of polymerization, a **cross-linker is used to fix functional monomers around template molecules**, thereby forming a highly cross-linked rigid polymer even after the removal of templates.
- The type and the amount of cross-linker have profound influences on the selectivity and binding capacity of MIPs.
- Usually, a too low amount of cross-linker will result in unstable mechanical properties due to the low cross-linking degree, and an extremely high amount of cross-linker will reduce the number of recognition sites per unit mass of MIPs.

Porogens

- Porogens (porogenic solvents) generally **act as dispersion media and pore forming agents** in the polymerization process.
- Usually, solvents used for MIP synthesis are 2-methoxyethanol, methanol, tetrahydrofuran (THF), acetonitrile, dichloroethane, chloroform, N,N-dimethylformamide(DMF) and toluene.
- The **polarity of porogens can affect the interaction between the template molecule and the functional monomer**, and therefore the adsorption properties of MIPs, especially in non-covalent interaction systems.
- Non-polar and less polar organic solvents, such as toluene, acetonitrile and chloroform, are often used for non-covalent imprinting to obtain good imprinting efficiency, since the adsorption properties and morphology of polymers are dependent on the types of solvents used.

Porogens

- To evaluate the selection processes of monomers and solvents for molecular imprinting and to have an insight into MIP selectivity, the use of theoretical calculations is very important.
- The effects of solvents on monomer–template binding energy using four solvents: acetone, acetonitrile, chloroform, and methanol.
- **Density functional theory (DFT)** had been used for all structural, vibrational frequency and solvent calculations.
- More recently, **room temperature ionic liquids (RTILs)** have been reported as an interesting class of solvents with unique characteristics.
- The **negligible vapor pressure of RTILs** can help **reduce the problem of MIP bed shrinkage** and they can also act as pore templates in the polymerization reaction.
- RTILs can accelerate the synthesis process, improving the selectivity and adsorption of trans-asconitic acid imprinted organic polymers.
- RTILs including [BMIM][BF₄], [BMIM][PF₆], [HMIM][PF₆] and [OMIM][PF₆] attained satisfactory selectivities and rebinding capacities for propranolol MIPs.
1-Butyl-3-methyl-imidazolium-tetrafluoroborate (BMIM BF₄)

Initiators

- MIPs are commonly prepared by **free radical polymerization (FRP), photopolymerization, and electropolymerization.**
- FRP can be initiated either thermally or photochemically for a wide range of functional groups and template structures.
- Aside from the peroxy compounds, azo compounds are extensively used as initiators
- Among them, azobisisobutyronitrile (AIBN) is most conveniently used at the decomposition temperatures of 50–70°C.
- To ensure the polymerization reaction, removal of the dissolved oxygen from polymerization solutions immediately prior to proliferation is very important.
- Oxygen can be cleared by bubbling an inert gas like nitrogen or argon.

Preparation procedures

- The selection of an appropriate preparation procedure is critical for the production of MIPs with desirable properties.
- Generally, the mechanisms of MIP preparation include **free-radical polymerization and sol-gel processes**.
- Free-radical polymerization is more popular and general.

Table 2 Comparison of different preparation procedures and imprinting methods of MIPs

| Mechanism | Imprinting method | Advantage | Disadvantage |
|-----------------------------|------------------------------|--|--|
| Free-radical polymerization | Bulk polymerization | Rapidity and simplicity in preparation; no requirement for sophisticated or expensive instrumentation; purity in the produced MIPs | Time-consuming of grinding, sieving; irregular particles in shape and size; low affinity sites |
| | Suspension polymerization | Simple process with one step polymerization; spherical particles | Big particle size (a few to a few hundred micrometres); poor recognition |
| | Emulsion polymerization | High yield, monodispersed polymeric particles; water-soluble polymers | Suffers from the presence of remnants of surfactants; low imprinting capacity |
| | Seed polymerization | Controllable regular spherical particles; mono-dispersity; suitable for HPLC | Laborious process; time-consuming |
| | Precipitation polymerization | One single preparative step; high-quality, uniform and spherical particles | Large amount of template; high dilution factor |
| Sol-gel process | Sol-gel | Ease of fabrication at room temperature; eco-friendly reaction solvent | Lack of polymerization method and the functional monomer |

Characterization methods

Table 3 Typical characterization methods and their main purposes for MIPs

| Purpose | Characterization method |
|---|--|
| Morphological evaluation | SEM, TEM, AFM |
| Screen of monomers for interaction with template or for the validation of computational design data | NMR, IR, UV-Vis |
| Structure analysis | X-ray absorption fine structure, diffraction and XPS |
| Measure the specific surface areas and pore sizes of the polymers | Nitrogen adsorption |
| Thermal stability evaluation | TGA |
| Magnetic property evaluation | VSM |

Smart MIT for MIPs

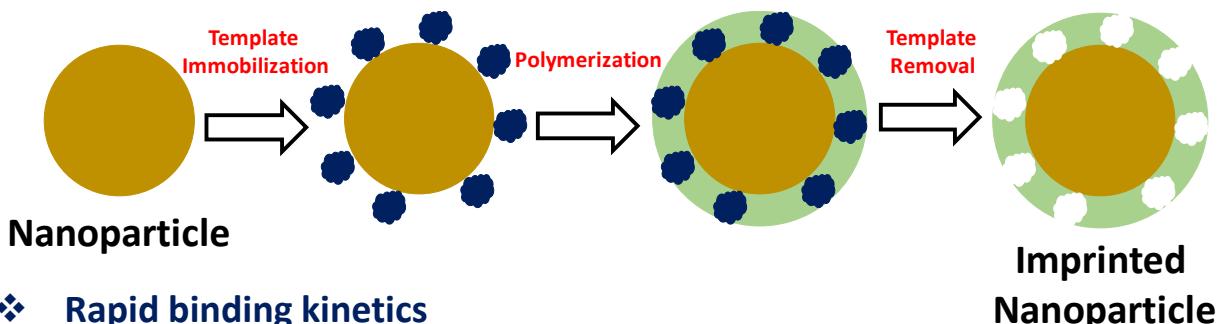
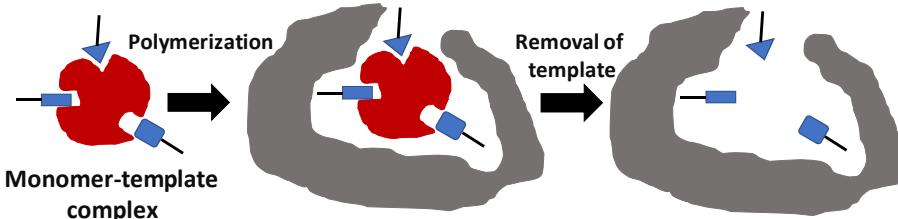
- MIPs still have many problems, such as template leakage, low binding capacity, irregular material shape, and incompatibility in aqueous media, which have greatly obstructed the applications of MIPs.
- As a result, various smart preparative technologies and strategies of MIT have emerged to cope with the problems.
- Smart MIT is classified into three main types, i.e., ingenious technologies (**surface imprinting technology**, **nanoimprinting technology**, etc.), special imprinting strategies (**the multitemplate/ functional monomer imprinting strategy**, **the dummy/segment imprinting strategy**, etc.) and **stimuli-responsive imprinting technologies** (magnetic/thermo-responsive technology, dual/multi responsive technology, etc.), have been developed.

Surface imprinting technology

- Mosbach, first reported the surface imprinting technology to prepare imprinted materials by **controlling templates to locate at the surface or in the proximity of materials' surface** to create more effective recognition sites.
- The complete removal of template molecules and good accessibility to target molecules,
- **Especially suited for imprinting macromolecules, such as proteins, cells and viruses**, since their large sizes usually hinder the templates from both leaving and rebinding the imprinted sites in traditional MIPs.
- For example, bovine serum albumin (BSA) as the template protein was immobilized on the surface of silica nanoparticles to synthesize the surface modified MIPs.
 - The results showed excellent selectivity and recognition ability for the protein template, as many specific recognition sites for the protein template were generated on the surface of MIPs.

Surface Molecular Imprinting

- Classical approach:
incorporate template and
monomers all together
- Difficulty in removing template



- ❖ Rapid binding kinetics
- ❖ Traces of template being easily removed
- ❖ More flexible incorporation of multifunctionality
- ❖ High surface-to-volume ratio, High level of specific binding sites
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**“Lock-key”
Molecular lock**

Nanoimprinting technology

- Molecular imprinting nanotechnologies has attracted considerable research interest in that **nanostructured MIPs (N-MIPs)** show significantly improved characteristics in contrast to bulk MIPs
- N-MIPs have higher surface area-to-volume ratios, providing a good accessibility to target species and leading to the improvement of binding kinetics and binding capacity.
- The templates located within x -nm from the surface can be removed from the bulk materials with a scale of d , and the resultant effective volume of imprinted materials that can rebind target $[d^3 - (d - 2x)^3]$
- In general, the x value is very small for bulk materials.
- When the imprinted materials with the same size were prepared in the form of nanostructures with a scale of $2x$ nm, all of the templates can be completely removed from the highly crosslinked matrix, and the resultant sites are all effective for target species.

Nanoimprinting technology

- Thus, nanoimprinted materials **are expected to improve the binding capacity, binding kinetics, and site accessibility of imprinted materials** N-MIPs with different forms such as nanoparticles, nanotubes and nanowires have been synthesized by using different nanotechnologies
- Zhang et al. reported the surface imprinting of 2,4,6-trinitrotoluene (TNT) molecules at the surface of silica nanoparticles.
 - The uniform core–shell particles with TNT-imprinted polymer nanoshells had a high density of effective recognition sites, which was nearly 5-fold higher than that of traditional imprinted materials.
 - The results provided a new strategy for preparing nanosized imprinted materials.

Comparison of bulk MIPs and Nanostructured MIPs

Table 4 Comparison of the properties of bulk MIPs and nanostructured MIPs

| Bulk MIPs | N-MIPs |
|---|--|
| Low surface-to-volume ratio, difficult to elute | High surface-to-volume ratio, greater total active surface area per weight unit of polymer |
| Broad distribution of binding sites with varying affinity, high level of non-specific binding sites | Similar affinity for all binding sites, high level of specific binding sites |
| Insoluble material, difficult to process, bulk, batch-to-batch variability | Soluble nanoparticles well dispersing in solution, better control of manufacturing process |
| Difficult to access the empty cavities encased within rigid matrix | Imprinted cavities being more easily accessible to the templates, improving binding kinetics and facilitating the template removal process |
| High possibility of template leaking from the polymer | Traces of template being easily removed |
| Limited prospects for <i>in vivo</i> applications | Biological activity showing infinite prospects for <i>in vivo</i> applications |

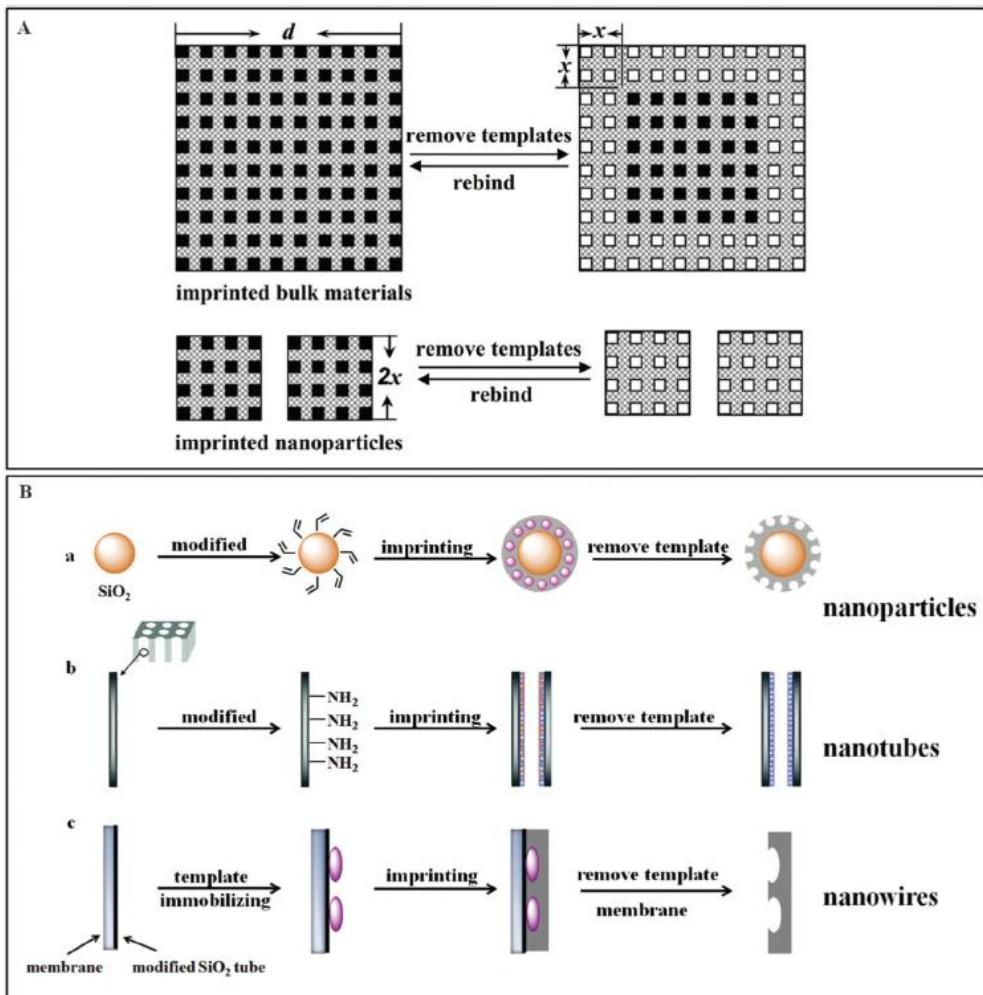


Fig. Schematic representation of nanoimprinting.

(A) Schematic illustration of the distribution of effective binding sites in the imprinted bulk materials and the nanosized, imprinted particles after the removal of templates.

(B) Schematic diagrams of nanoimprinting process for different forms of N-MIPs. (a) Imprinting on the SiO_2 support for the formation of core–shell imprinted nanoparticles. SiO_2 core particles were first modified with the vinyl functional monomer, followed by initiating an imprinting polymerization reaction, leading to the formation of imprinted shells at the surface of silica particles.

(b) Imprinting on silica nanotubes for the formation of imprinting nanotubes. SiO_2 nanotubes were first modified with APTES, followed by the sol–gel process, leading to the formation of imprinted shells at the surface of SiO_2 nanotubes.

(c) Imprinting on a sacrificial membrane support by employing an immobilized protein template approach for the formation of imprinted nanowires. The template molecule was firstly immobilized on the inner wall of a porous alumina membrane, followed by imprinting polymerization reaction, leading to the formation of nanowires by removing supporting alumina.

Special imprinting

- Some special imprinting strategies have emerged, such as multi-template imprinting, multi-functional monomer imprinting, dummy imprinting and segment imprinting strategies

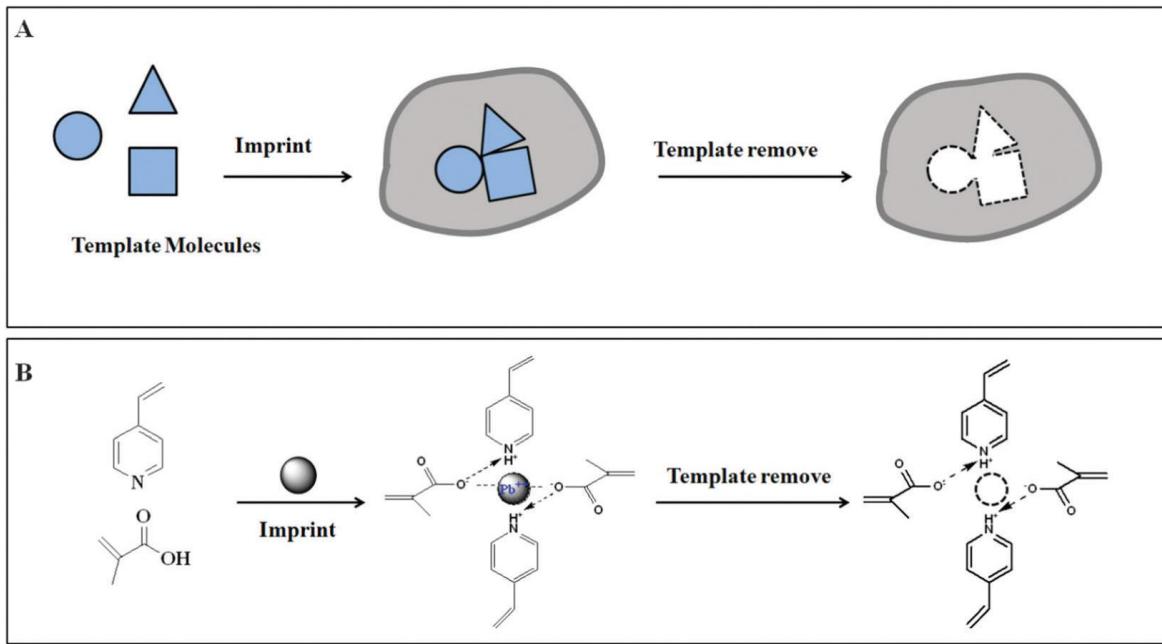
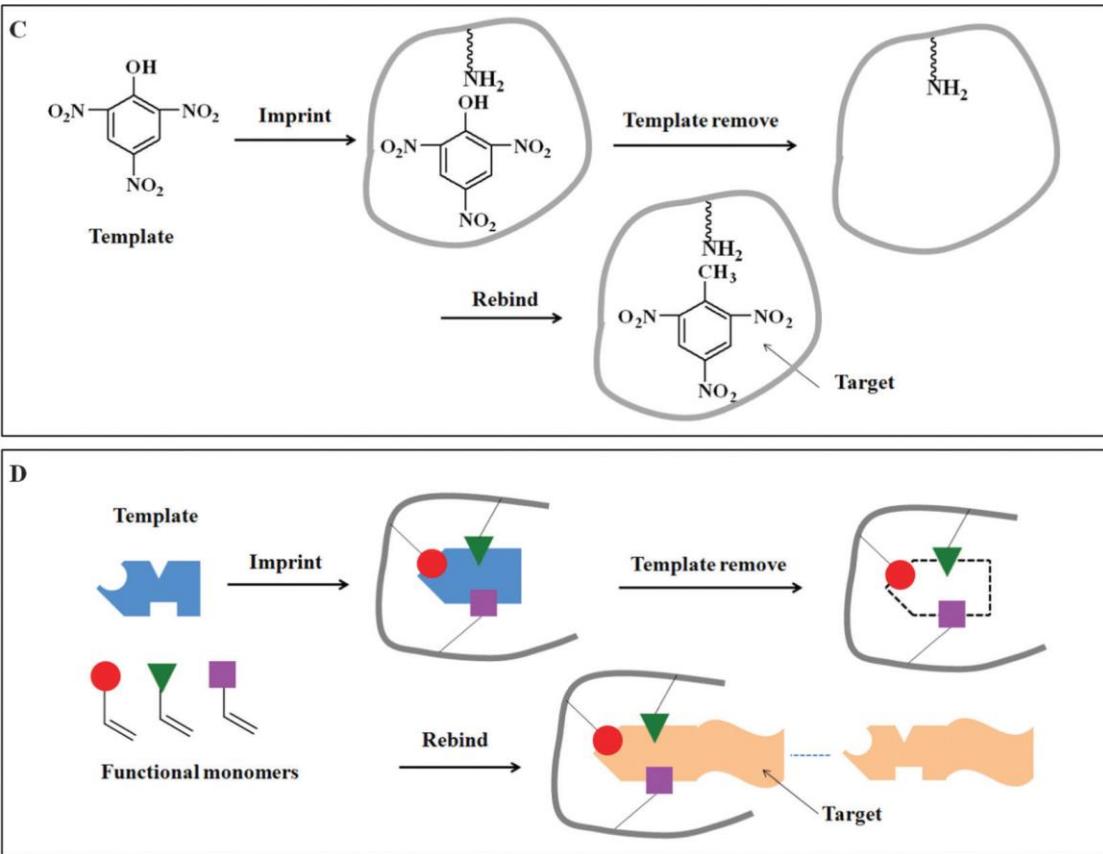


Fig. representation of special imprinting. (A) Schematic diagram of multiple template imprinting for multi-analyte binding. (B) Schematic diagram of multiple functional monomers imprinting for Pb^{2+}

Special imprinting



(C) Schematic diagram of **dummy imprinting**. Using trinitrophenol (TNP) as the dummy template for determination of 2,4,6-trinitrotoluene (TNT) based on the fact that TNT has a similar structure as TNP. (D) Schematic diagram of **fragment imprinting**. Using part of target molecular as the template, recognition sites were created by the removal of the template molecule, the obtained recognition sites can bind the whole target molecule based on the fact that target molecule has a fragment structure like the template molecule

Stimuli responsive MIPs.

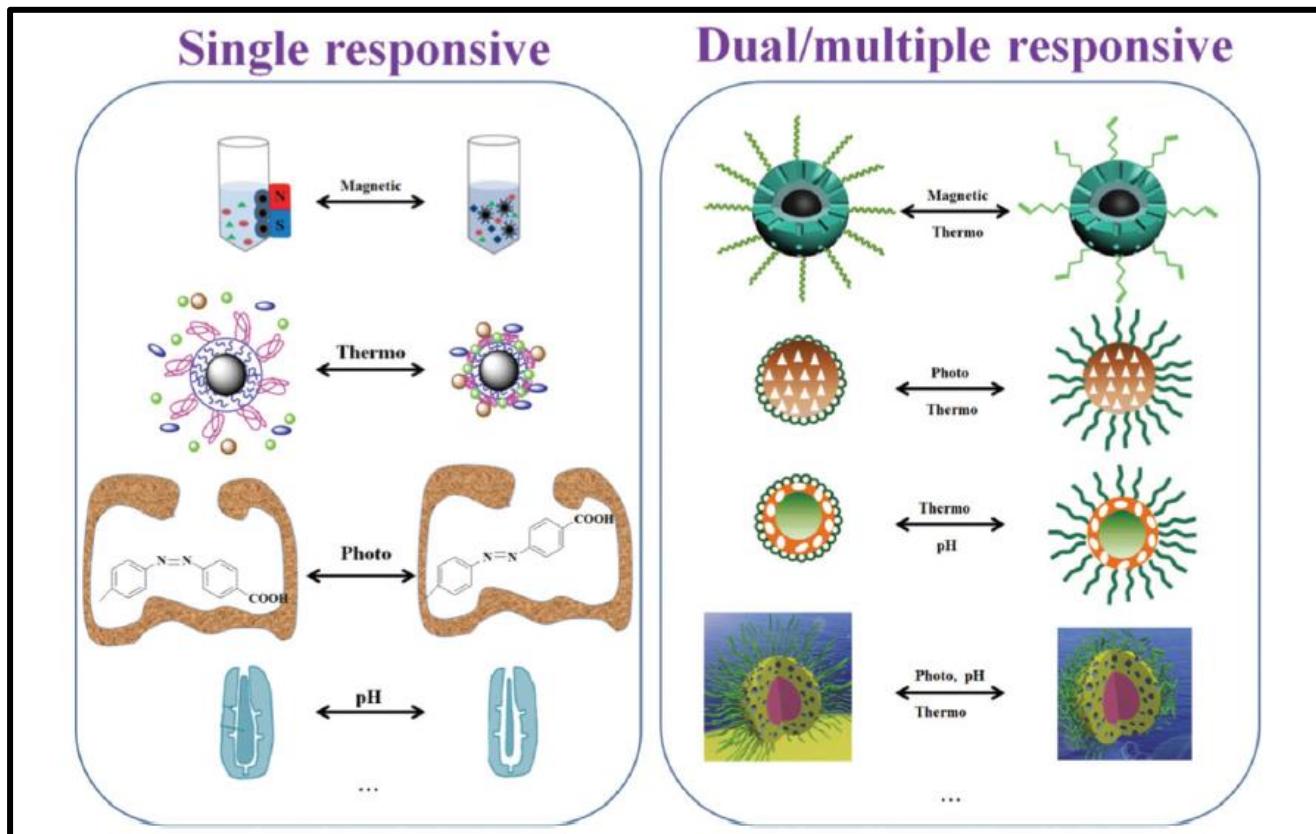


Fig. 11 Schemes of stimuli responsive MIPs. SR-MIPs are divided into two main types: **single responsive MIPs** and **dual/multi responsive MIPs**. Single responsive MIPs mainly include magnetic responsive using Fe_3O_4 as response unit, thermo-responsive using N-isopropylacrylamide (NIPAAm) as response unit; photo-responsive using azobenzene as response unit and pH responsive MIPs using poly(acrylic acid) as response unit; etc. Dual/multi responsive MIPs mainly include magnetic/thermo responsive MIPs using Fe_3O_4 and NIPAAm as response units, photo/thermo responsive MIPs using azobenzene and NIPAAm as response units; thermo/pH responsive MIPs using NIPAAm and 4-vinylphenylboronic acid (p-VPBA) as response units; thermo/photo/pH responsive MIPs using azobenzene, NIPAAm and 4-((4-methacryloyloxy)-phenylazo)benzoic acid (MPABA) as response units.

Applications of MIPs

Sample pre-treatment: (selective sorbent materials)

- Especially for trace/ultra trace level analytes, samples should be enriched or transformed into optimal forms prior to the final instrument analysis.
- Choosing the appropriate sample pre-treatment techniques plays an important role in qualitative and quantitative determination.
 - Liquid liquid extraction (LLE) is the most widely used pre-treatment technique while it has the disadvantages of large organic solvent consumption and low enrichment efficiency.
 - In order to overcome those drawbacks, a series of extraction techniques have been developed, such as liquid phase microextraction (LPME), solid phase extraction (SPE), solid phase microextraction (SPME) and stir bar sorption extraction (SBSE).
- However, these extraction, lack selectivity and suffer from matrix interferences
- Fortunately, MIPs have outstanding recognition performances towards target template molecules, and therefore they can be utilized as **selective sorbent materials** in the customized pretreatment techniques.

Applications of MIPs

Chromatographic separation:

- MIPs are also used as stationary phases in chromatography techniques, such as HPLC, capillary electrochromatography (CEC), capillary LC (CLC) and thin layer chromatography (TLC), as packing materials and monolithic column materials, due to their high affinity and selectivity to the target analytes.

Chemical and biological sensing

- The employment of MIPs as specific sensing materials in sensors, namely MIP-based sensors, which were first proposed by Mosbach to monitor the **specific binding of vitamin K1 to a “surface-imprinted” silicon surface by using optical surface ellipsometry**, has made considerable progress due to their high selectivity and stability, simplicity, cost-effectiveness, and versatility in the fields such as clinical diagnostics, environmental control, food analysis and drug screening.
- The characteristic feature of MIP-based sensors is that the MIPs have **both recognition and transduction properties**, that is, the MIPs as recognition elements can specifically bind target analytes and as transduction elements can generate output signals for detection.
-

Applications of MIPs

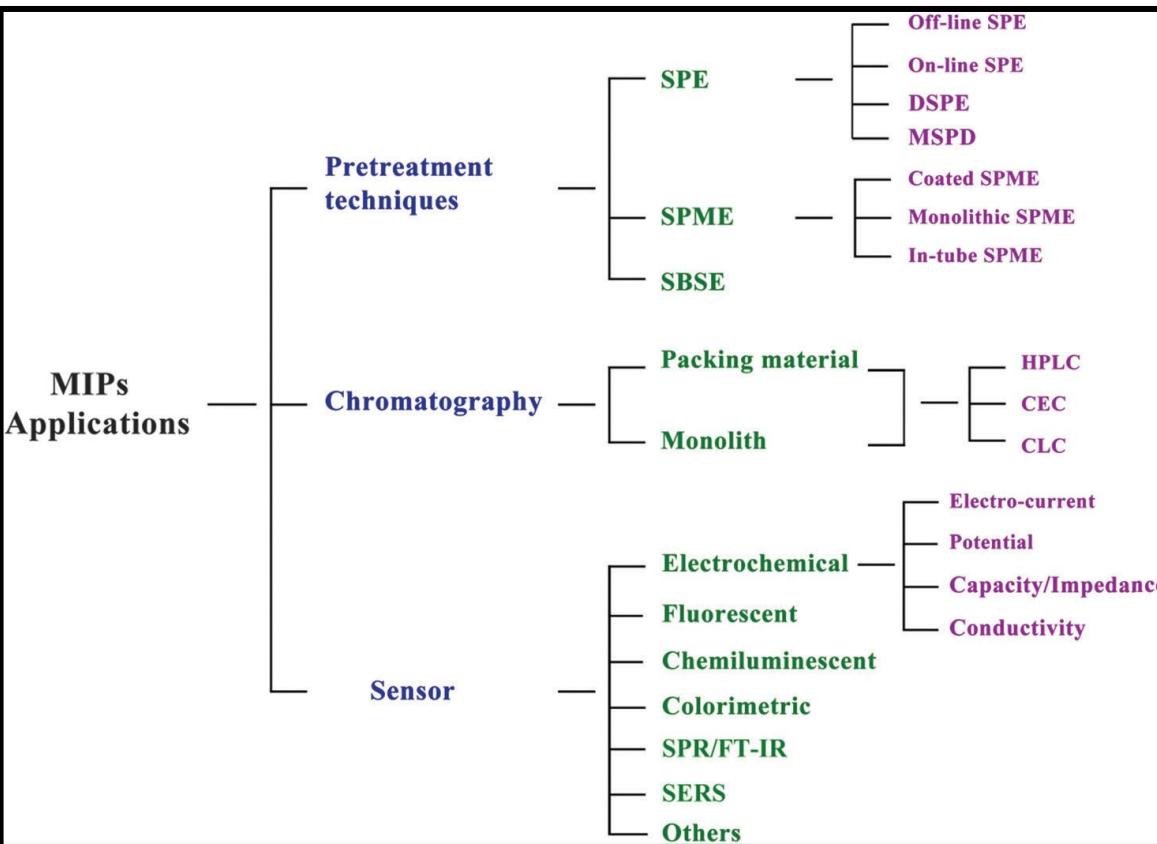


Fig. Structural diagram of the applications of MIPs in pre-treatment techniques, chromatography and sensor. Abbreviations: SPE, solid phase extraction; DSPE, dispersive SPE; MSPD, matrix solid phase dispersion; SPME, solid phase microextraction; SBSE, stir bar sorption extraction; HPLC, high performance liquid chromatography; CEC, capillary electrochromatography; CLC, capillary liquid chromatography.

Applications of MIPs

- Imprints against the **bronchodilator drug theophylline** and against the tranquilizer diazepam have shown amazingly specific recognition
- In fact, when these MIPs were tested in competitive radioimmuno- style assays, their **recognition of related structures was either nonexistent or far below that of the original print molecule.**
- Amazingly, the **cross-reactivity profiles of these MIPs was practically identical to those reported for monoclonal antibodies** against these drugs.
- The anti-theophylline MIPs were used for the determination of theophylline concentrations in patient serum samples, pointing towards their use as stable alternatives to antibodies

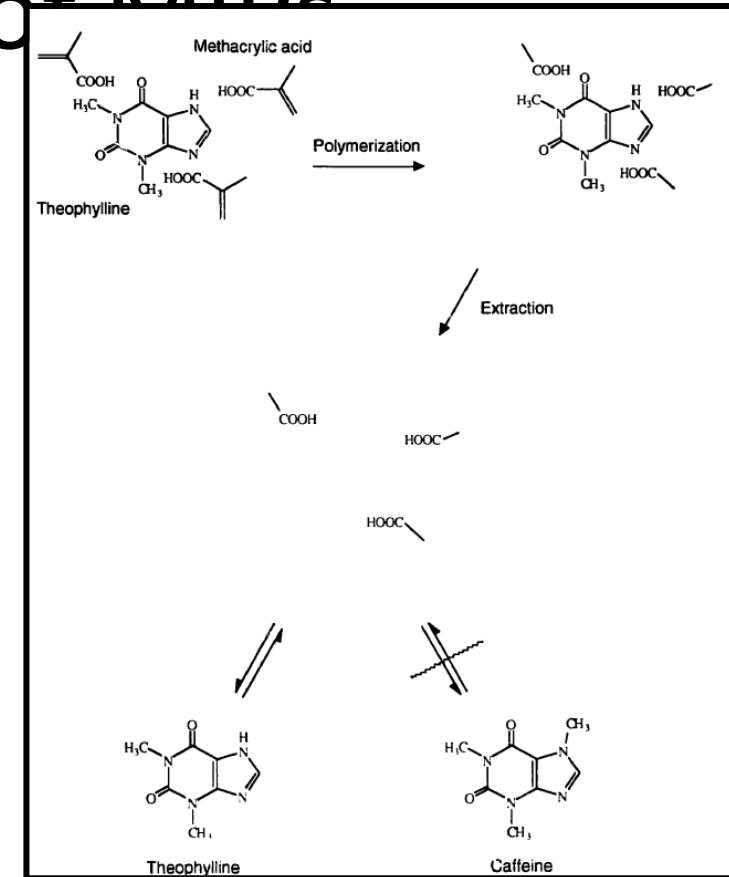


Figure: Schematic representation of the preparation of antibody-binding-site mimics. Theophylline, the print molecule, binds effectively to the imprint sites, whereas the structurally closely related caffeine does not.

Protein imprinting

- the specific binding of the glycoprotein transferrin on imprints obtained by surface imprinting

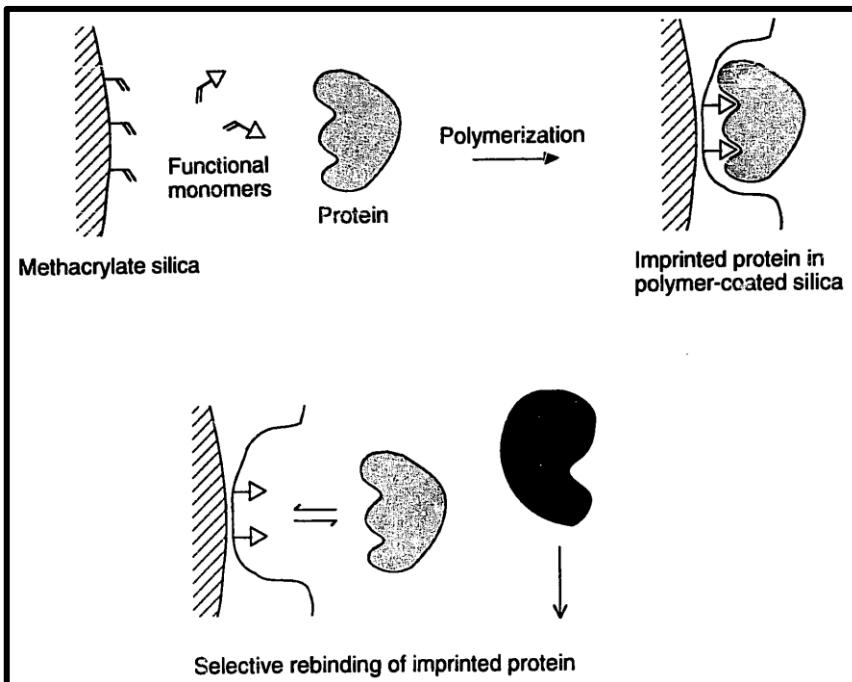
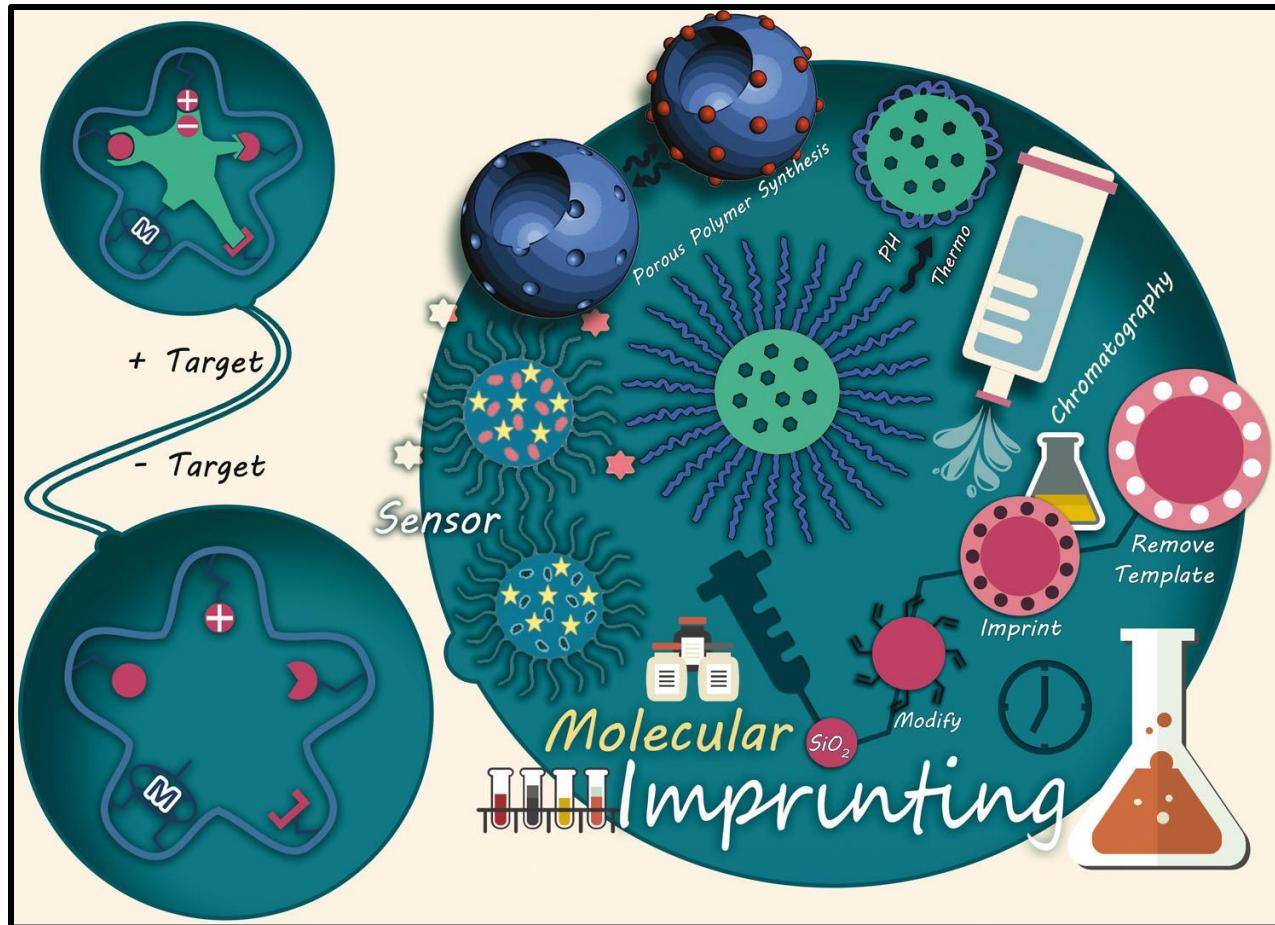
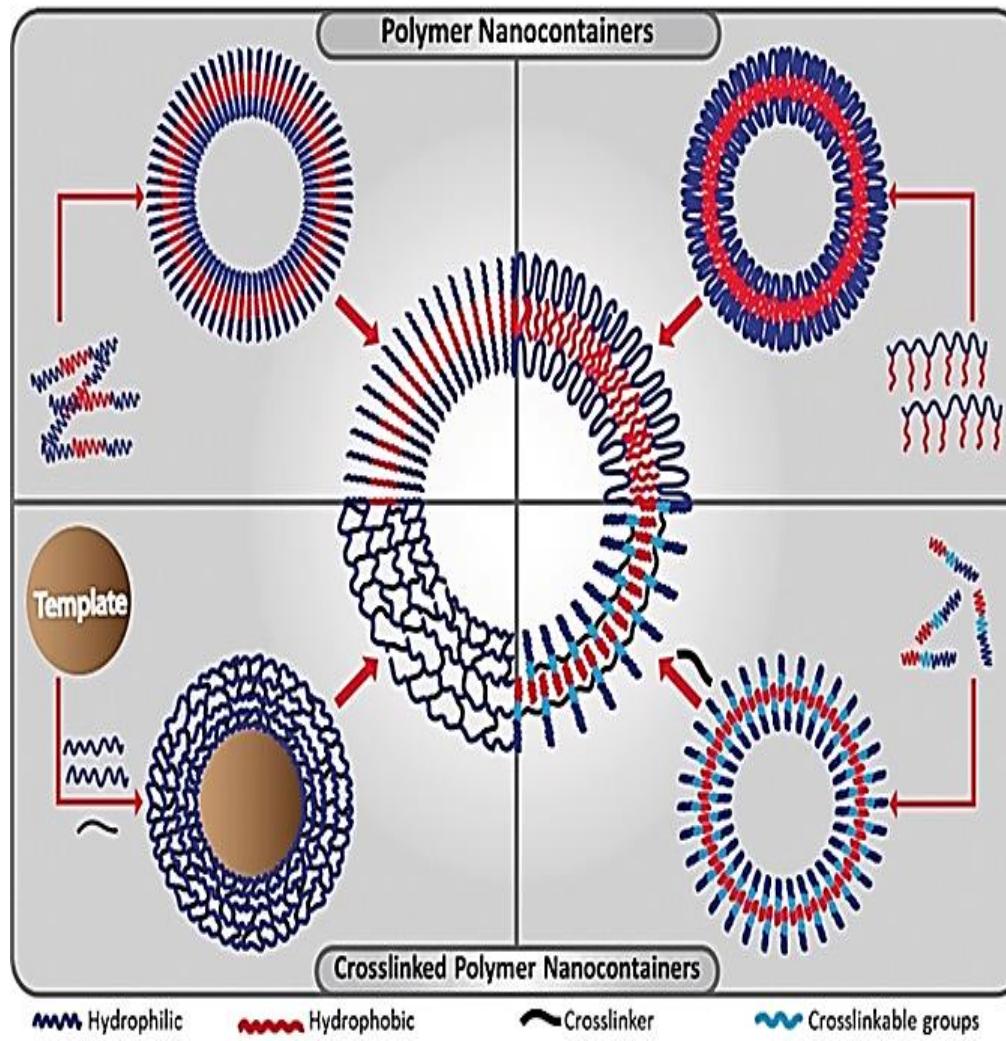


Fig: Highly schematic presentation of the concept utilized in a protein imprinting protocol. After allowing the protein to interact with functional monomers, the protein is 'surface imprinted' to a carrier. Subsequent protein recognition is specific for the print protein, owing to the complementary binding sites.



Thank You!!



Polymer NanO-containeRS

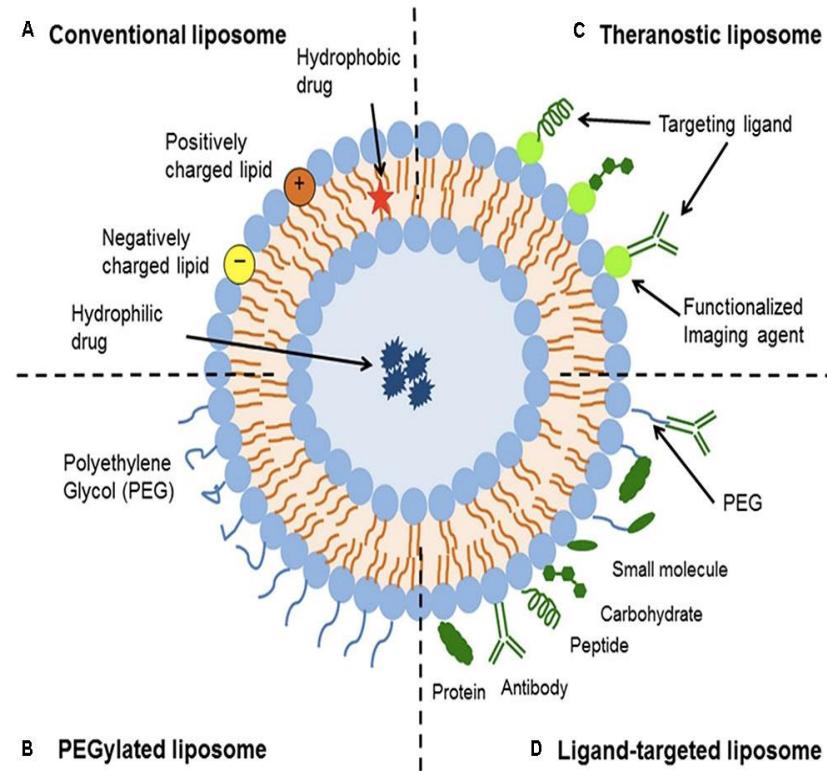
Intracellular Delivery Systems

Introduction

- Hollow polymer particles – the so-called “polymer nanocontainers”
- High stability and tunable properties.
- High potential for applications in biotechnology:
 - a) confined reaction vessels.
 - b) protective shells for enzymes.
 - c) ‘traps’ for the selective recovery of biotransformation or polymerase chain reaction products.
- Major interest as drug delivery devices in biomedical field.

From Liposomes in Biotechnology to Polymer Nanocontainers in Therapy

- The pioneering studies on lipid vesicles or liposomes were conducted in the early 1960s.
- Vesicles are spherically closed lipid bilayers that result from the naturally occurring self-assembly process of amphiphilic molecules.
- Liposomes that served mainly as model systems to study biological membranes can be used as transport vehicles for drugs.
- In topology of two-dimensional surfaces floating in a three-dimensional continuum.



From Liposomes in Biotechnology to Polymer Nanocontainers in Therapy

- As model systems in biophysics (properties of cell membranes and channels).
- In chemistry (catalysis, energy conversion and photosynthesis).
- Colloid science (stability and thermodynamics of finite systems).
- Biochemistry (function of membrane proteins).
- And in biology (excretion, cell function, trafficking and signaling, gene delivery and function).

From Liposomes in Biotechnology to Polymer Nanocontainers in Therapy

- Controlled delivery devices for drugs (example antifungals, anticancer agents, vaccines),
- Nonviral gene delivery vectors,
- Cosmetic formulations(skin-care products, shampoo),
- Diagnostic tools.
- ‘Stealth’ liposomes- improvements in their formulation, mainly to increase their stability and interaction characteristics.

From Liposomes in Biotechnology to Polymer

- Biotechnological applications of liposomes are based on the compartmentalization:
Nanocontainers in Therapy
- For example vesicles from 2,4-tricosadiynoic acid (TCDA) as the lipid matrix and dioctadecyl glyceryl ether- β -glycoside as a receptor to detect *Escherichia coli*.
- Served as effective colorimetric biosensors.
- due to the diacetylene groups, appear blue.
- Binding to bacteria mechanical stress inside the vesicular membranes change in effective conjugation length vesicle dispersion turns red.



From Liposomes in Biotechnology to Polymer Nanocontainers in Therapy

- Liposome: DNA amplification by PCR and minimal cell bioreactors to express proteins (Oberholzer et al.).
- Concept of “Artificial cell”
- DNA replication or ribosomal synthesis of polypeptides can be carried out inside the compartment offered by the aqueous pool of the liposomes.

Drawbacks:

- inherent colloidal and biological instability
- very short lifetimes.

Dendrime

- Dendrimers are highly branched polymers with radial symmetry and uniform size, which adopt a globular shape in solution.
- Dendritic macromolecules or starburst dendrimers consist of three different structural or topological units that result from an iterative reaction sequence:
- a central core from which the repetitive branching units extend/emanate radially to finish in the outer layer of end-groups.

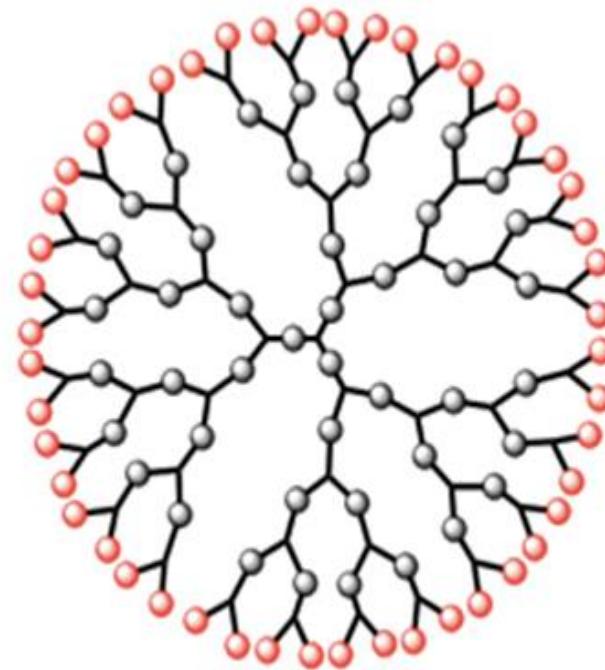
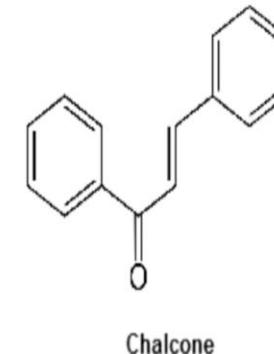
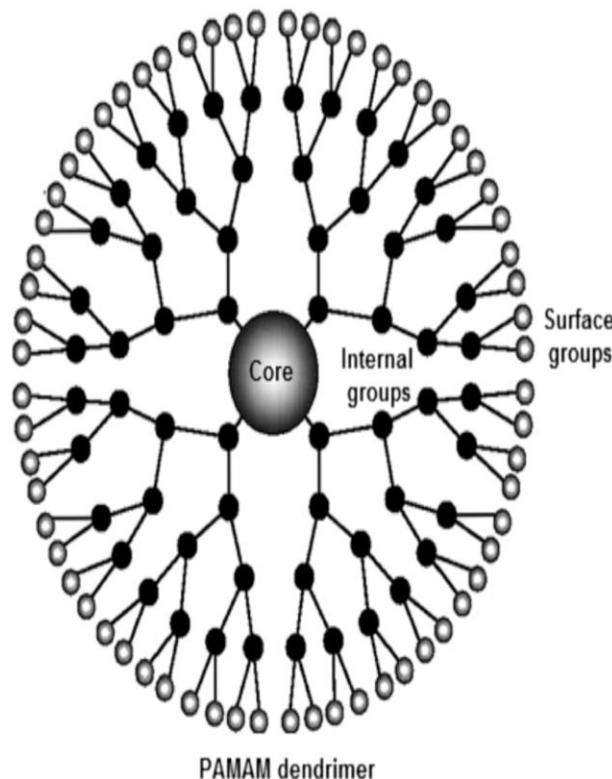


Fig. 1 Basic structure of dendrimer

Dendrimers

- With each generation- the density is increased due to the geometric growth at each branching point.
- Molecules have been encapsulated during the synthesis of the dendrimer and were retained within the central part of the macromolecules.
- The release of the molecules could be facilitated by an appropriate modification of the external groups of the dendrimer.
- Synthetic design affords dendrimers with tailored structures
- Polyamidoamine (PAMAM) dendrimers are most common class of dendrimers suitable for various applications.



Dendrimers

- The repeating units in the interior determine the solubilization properties towards guest molecules.
- The functional terminal groups influence the solubility of the dendrimer itself in a given solvent.
- Amphiphilic dendrimers - in which the interior is comprised of hydrophobic moieties and the external groups consist of hydrophilic units.
- Also, regarded as “unimolecular micelles”.
- Dendrimers can be used to prepare real hollow structures by selectively crosslinking their outer shell and degrading the original core region.

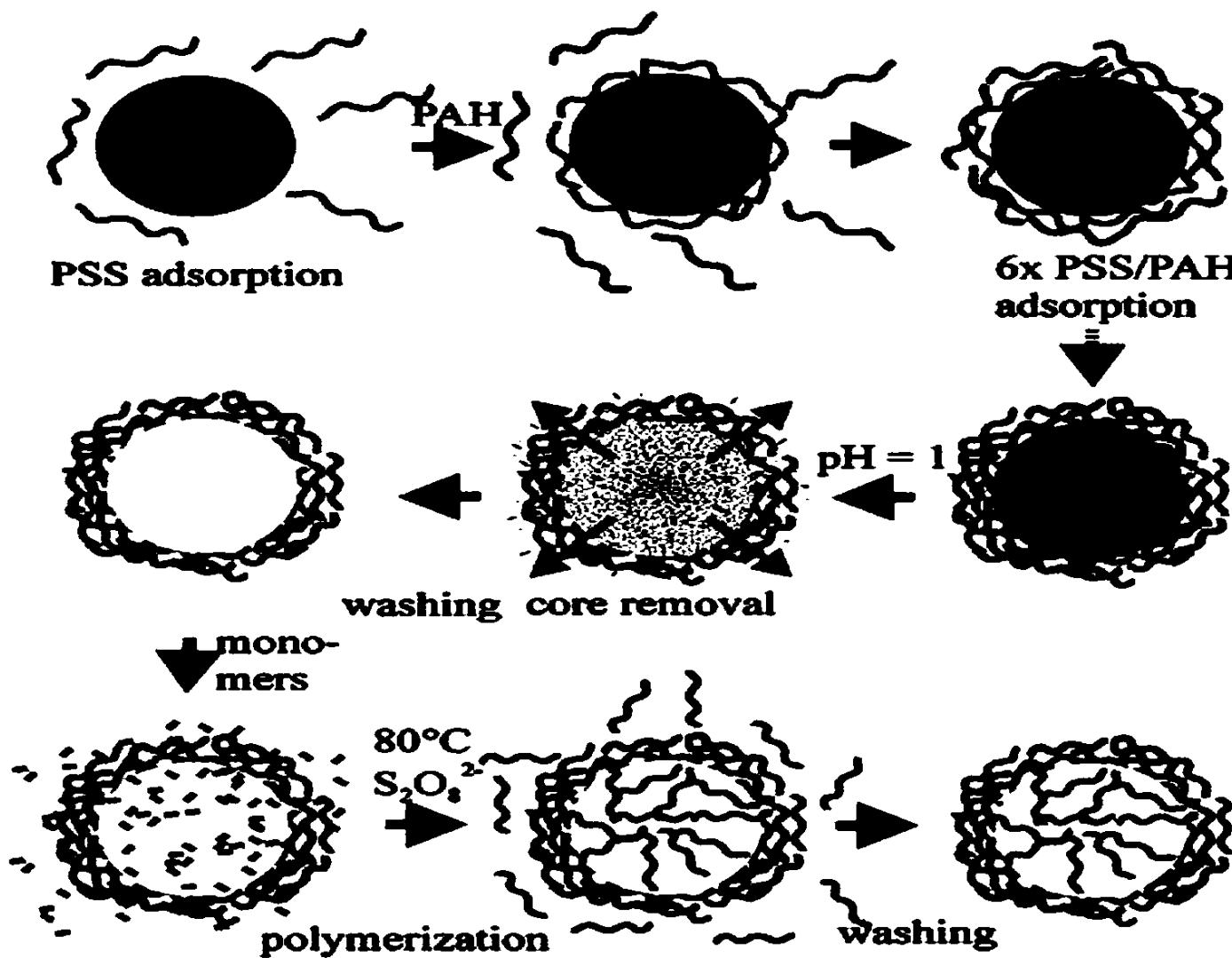
Polymer Nanocontainers (Contd.) :

Layer by Layer (LbL) Deposition

- Polyelectrolyte self-assembly at charged surfaces to produce polymer capsules.
- By use of layer-by-layer (LbL) deposition steps of oppositely charged polyelectrolytes.
- The driving force behind the LbL method at each step of the assembly is the electrostatic attraction between the added polymer and the surface.
- Polyelectrolyte molecules having the opposite charge (e.g., polycations) are readily adsorbed due to electrostatic interactions with the surface.

Layer by Layer (LbL) Deposition

- All of the ionic groups of the adsorbed polyelectrolyte are not consumed by the electrostatic interactions.
- The original surface charge is usually overcompensated by the adsorbed polymer.
- Thus the surface charge of the coated particle changes its sign.
- Which is further available for the adsorption of a polyelectrolyte of again opposite charge (i. e., a polyanion).



Preparing hollow spheres using layer-by-layer deposition of oppositely charged polyelectrolytes on colloidal particles and subsequent encapsulation of polymers in a “ship in a bottle” fashion. PSS: sodium polystyrene sulfonate; PAH: poly(allylamine) hydrochloride.

Layer by Layer (LbL) Deposition

- Particles with diameters ranging from 0.2 to 10 μm has been reported.
- Thickness of the layered shell is determined by the number of polyelectrolyte layers.
- A variety of charged substances, such as synthetic polyelectrolytes, biopolymers, lipids, and inorganic particles have been incorporated as layer constituents.
- As a template for this approach, mainly colloids
- Polystyrene latexes or melamine formaldehyde particles, gold and proteins have been used as templates

Layer by Layer (LbL) Deposition

- Biopolymers like alginate and polylysine can also be used in a similar way to yield biocompatible nanocapsules.
- Uncharged hydrophobic compounds could be also encapsulated using the LbL technique.
- Core formed by uncharged low molecular-weight microcrystalline substances (pyrene and fluorescein diacetate).
- dispersed in water via micellization with amphiphilic substances such as ionic surfactants, phospholipids, or amphiphilic polyelectrolytes.

Layer by Layer (LbL) Deposition

- A LbL procedure of depositing layers of polyelectrolytes rendered stabilized core shell particles.
- The release of the encapsulated substances followed by the intrinsic fluorescence of the core forming material, was triggered by the addition of ethanol.
- Wang et al. designed biologically active polymer microcontainers using the LBL assembly.
- They functionalized luminescent polymer containers with anti-immunoglobulin G which rendered them biospecific via their IgG partners.

Layer by Layer (LbL) Deposition

- These quantum dot-tagged beads open new opportunities in a range of biotechnological applications.
- quantum dots exhibit higher photo-bleaching threshold, quantum yield, and chemical stability than their organic fluorophore analogs.
- their spectral properties can be fine-tuned by controlling their size and, similar to planar LbL luminescent films.
- crosslinked luminescent core-shell particles and hollow capsules could also be used as light-emitting devices.

Layer by Layer (LbL) Deposition

- use in the area of biosensing.
- The concept of artificial cells has been also applied to polyelectrolyte microcapsules.
- Tiourina et al. used hollow microcapsules fabricated by stepwise adsorption of polyelectrolytes and phospholipids as so-called artificial cells.
- Showed high permeability for ions.
- ion-channel-forming peptides such as gramicidin and valinomycin were incorporated into the lipid-polymer composite shell of the microcapsules resulting in membrane potential comparable to biological cells

Block Copolymer Self-Assembly

- Amphiphilic block copolymers (which are polymers consisting of at least two chemically different parts, hydrophobic versus hydrophilic or rod versus coil) may self-assemble into various lyotropic mesophases (liquid-crystalline mesophases) .
- Potential for encapsulating large quantities of guest molecules within their central cavity.
- Block copolymer chemistry allows the introduction of a wide variety of different block structures.
- Considerably more stable due to their larger size, slower dynamics, and inherent steric stabilization.

Block Copolymer Self-Assembly

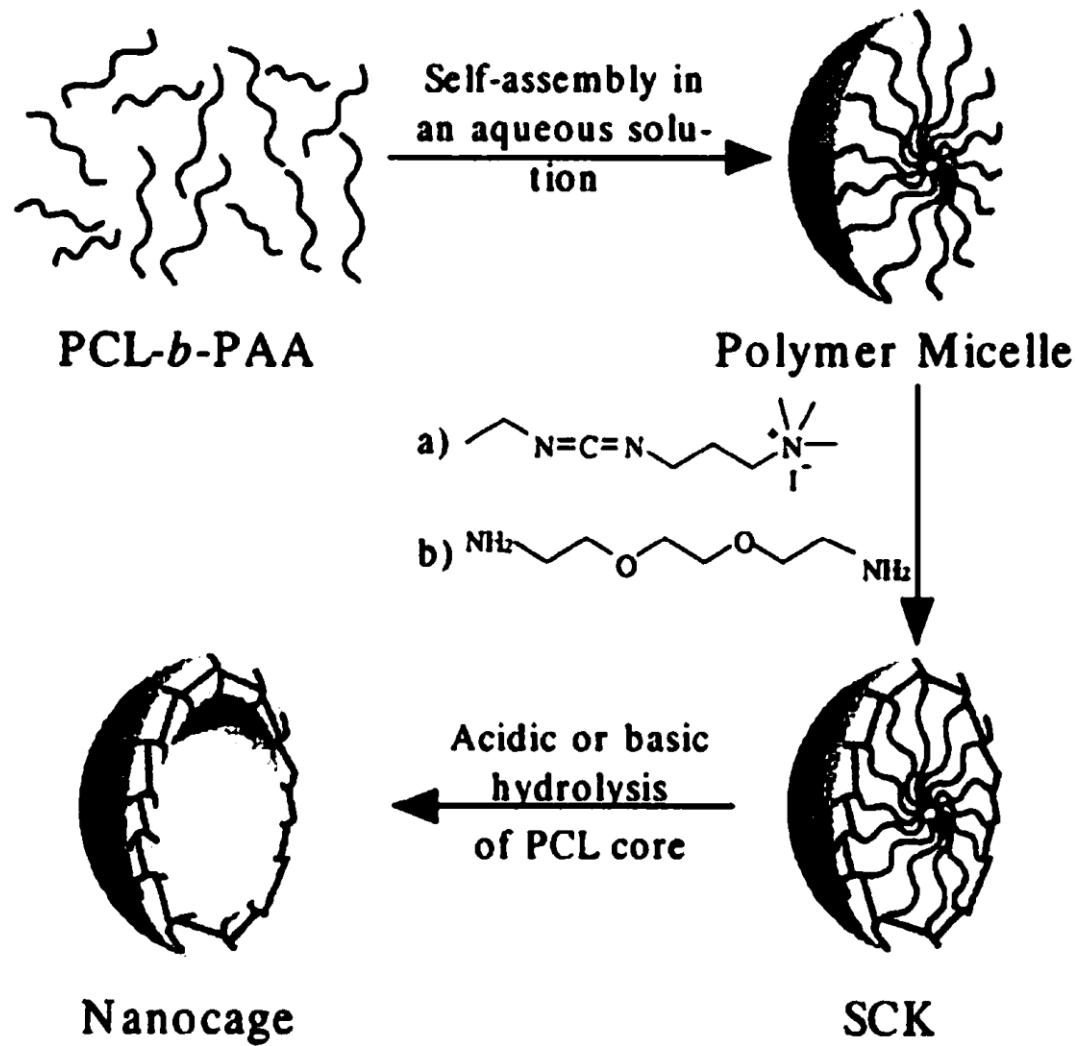
- Depending on their block length ratio, the critical aggregation concentration (c. a. c.) of these polymers can be shifted to extremely low values.
- Which makes superstructures resistant against dilution – an essential requirement for medical applications

Shell Cross-linked Knedel's (SCKs)

- Special type of nanoparticles having core-shell morphology.
- These systems are formed by aggregation of amphiphilic di- and triblock copolymers into micelles.
- An intra micellar crosslinking of the corona-forming blocks leads to the highly stable so-called SCKs, with sizes ranging from 50 to 250 nm.
- In a second step, the backbone of the core forming blocks can be cleaved.

Shell Cross-linked Knedel's (SCKs)

- The low molar-mass degradation products extracted.
- Leaving behind nanocages formed by a crosslinked polymer shell.



General procedure for the preparation of hollow shell cross-linked knedels (SCKs) nanocages from amphiphilic Di block copolymers. poly(ϵ -caprolactone)-block-poly(acrylic acid)-block-poly(acrylamide)

- micelles formed from amphiphilic diblock copolymers for the solubilization of hydrophobic drugs.
- In aqueous solution, the hydrophobic blocks form the micellar core while the hydrophilic ones build the corona.
- The core serves as a microenvironment for the lipophilic drugs, while the outer shell serves as a stabilizing interface between the hydrophobic core and the external medium.
- Example 1 : Meier's group has described the spontaneous formation of vesicles resulting from the self-assembly of a poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA) triblock copolymer

Block Copolymer Nanocontainers

- polymer was additionally modified with reactive methacrylate groups at the ends of the hydrophilic blocks.
- shape-persistent polymer nanocontainers were formed due to free radical polymerization of these methacrylate end groups.
- diameters ranging from 50 to 250 nm.
- Example 2: amphiphilic ABC triblock copolymers, with two different water-soluble blocks A and C (A = poly(2-methyl oxazoline), PMOXA; B = poly(dimethyl siloxane), PDMS; C = polyethylene oxide, PEO).

Block Copolymer Nanocontainers

- Similar nanospheres with superior properties inherent to the asymmetry of their membrane were obtained.
- Extremely high mechanical stability with high flexibility provided by the hydrophobic PDMS middle blocks.

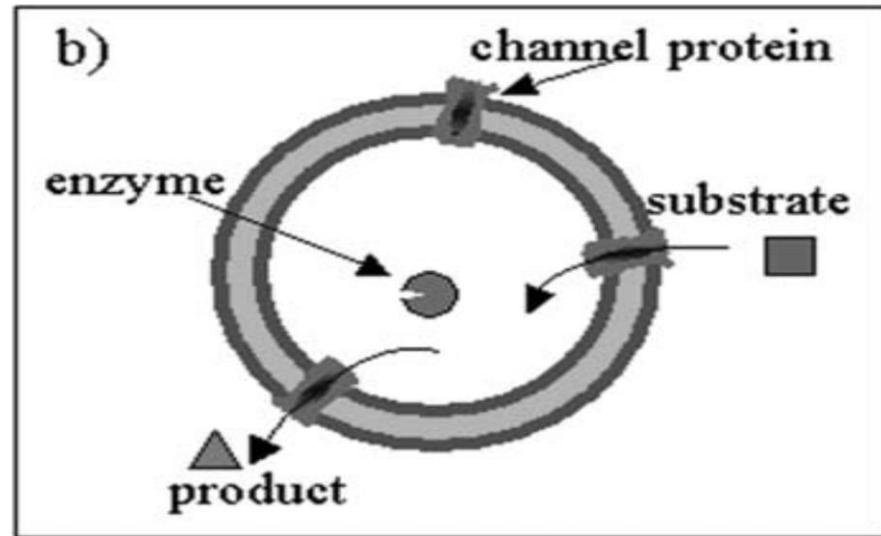
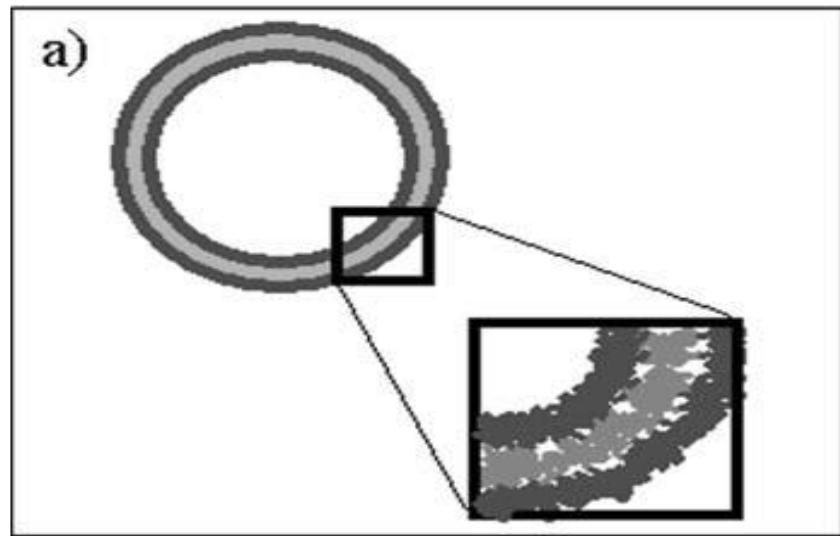
Block Copolymer Nanocontainers

Block Copolymer Protein Hybrid Systems (Polymer nanocontainer with controlled permeability)

- A new type of hybrid material emerged from the combination of biological molecules and block copolymers.
- A new class of biologically “active” **super-amphiphiles** composed of a block copolymer and an enzyme was designed.
- Consisting of an enzyme head group and a single covalently connected hydrophobic polymeric tail.
- Obtained by the coupling of maleimide-functionalized polystyrene to a reduced lipase.

Block Copolymer Protein Hybrid Systems

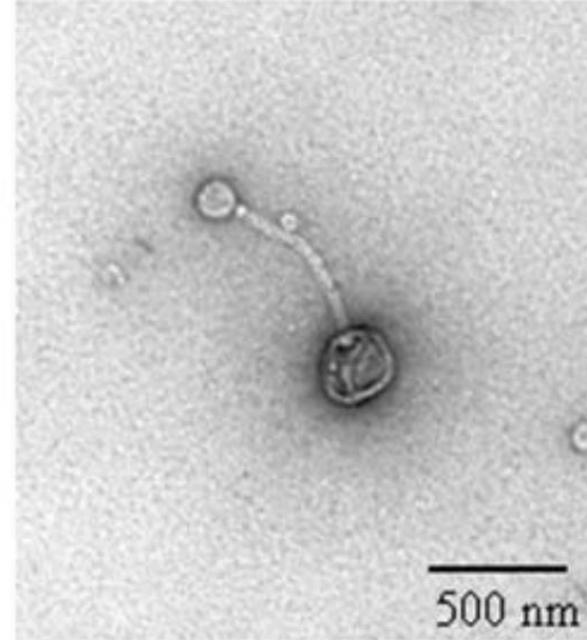
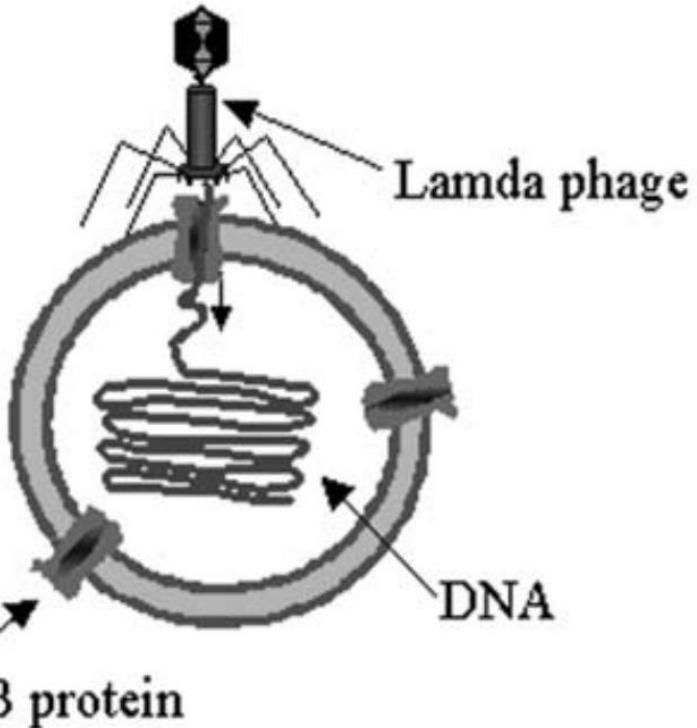
- lipase remained functional in the self-assembled superstructures of these ‘**Super amphiphiles**’.
- A pH-sensitive hybrid material produced by the formation of polymer vesicles or “**peptosomes**” by the self-assembly of poly(butadiene)-block-poly(L-glutamate) in dilute aqueous solution.
- Poly(L-glutamate) performs a pH-dependent helix-coil transition that does not alter the vesicle morphology.
- Due to their hydrophilic polypeptide chains, copolymer vesicles seemed to be particularly suited for biological applications.



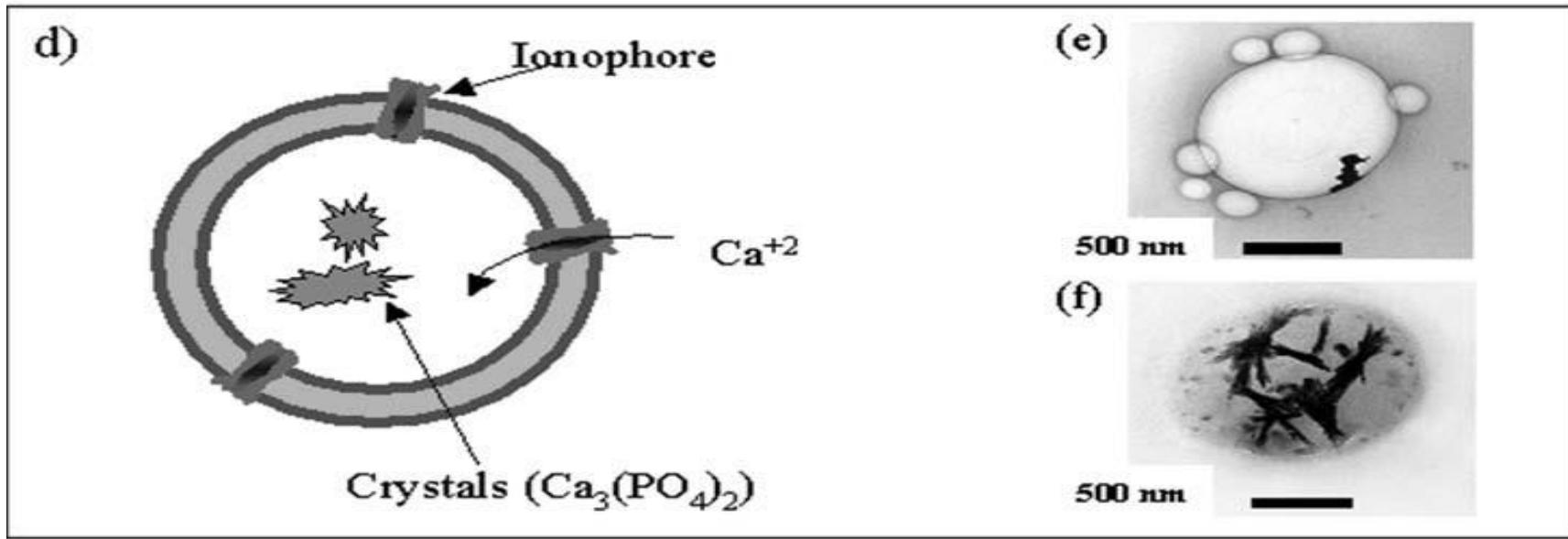
(a) Schematic view of an ABA triblock copolymer vesicle and magnification of the structure of its membrane, showing the constituting polymer chains.

(b) Representation of a BioNanoreactor with encapsulated β -lactamase and inserted membrane channel proteins to facilitate diffusion of substrates and products in and out of the nanoreactor.

c)



(c) Model of viral DNA encapsulation via phage binding and injection into nanocontainers, and transmission electron micrograph (TEM) showing the binding of a phage onto an ABA triblock copolymer vesicle.



(d) Schematic representation of an ABA nanocontainer with incorporated ionophores in its membrane used as biomineralization device. TEMs showing
(e) calcium phosphate crystals after 1 h and
(f) after 24 h.

Block Copolymer Protein Hybrid Systems

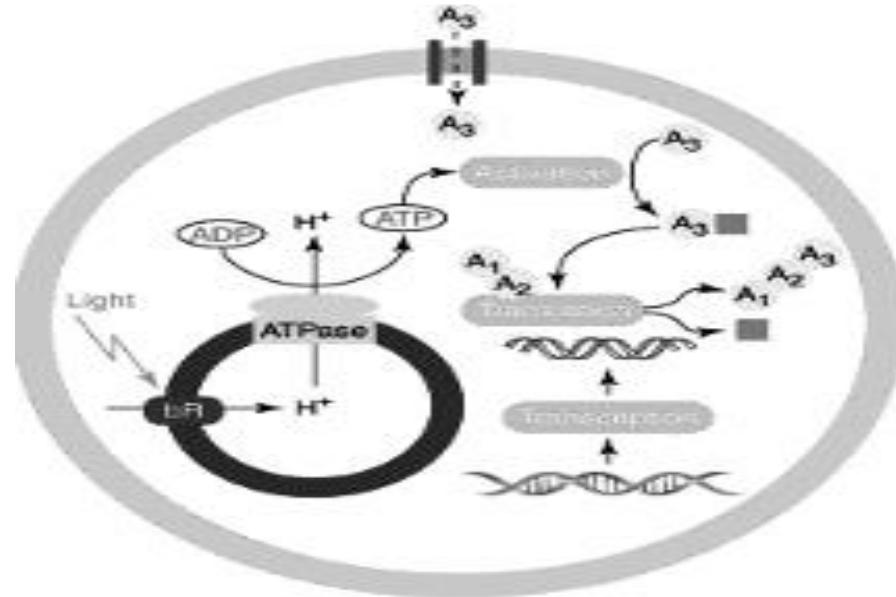
- Membrane receptors can also be incorporated into the walls of such polymer nanocontainers.
- access to the proteins could be controlled to a certain degree via the length of the hydrophilic blocks of the underlying amphiphilic block copolymers.
- longer hydrophilic chains, they are “hidden” below a hydrophilic polymer layer so that larger ligands had no access to them.
- Such receptors bearing channels provide as an elegant method to load polymer nanocontainers with DNA.

Block Copolymer Protein Hybrid Systems

- The electroneutrality and the low immunogenicity and toxicity of such DNA-loaded nanocontainers renders them highly interesting as new vectors for gene therapy.
- Receptor-bearing polymer nanocontainers may be of particular interest as biosensors.
- Major advantage of these systems is that an entire detection and signaling cascade can be incorporated into a single nanocontainer.
- Block copolymer nanocontainers can be regarded as miniaturized artificial cells allowing for massive miniaturization.

Block Copolymer Protein Hybrid Systems

- high mechanical and(bio)chemical stability, the polymer containers provide a constant environment for encapsulated analytic molecules



Hypothetical representation of a cell-like structure in which DNA is transcribed to RNA and translated to the protein via an encapsulated transcription and translation systems.

- Stimuli-responsive peptides and proteins incorporated in the walls of polymer nanocontainers can be used as “switches” to control molecular exchange across polymer membranes.
- Such systems can be regarded as mimetics of virion cages, which show a structural transition that leads to the opening of gated pores within the virus shell upon pH changes.
- Can be obtained by core-shell emulsion polymerization.
- A two-step polymerization led to crosslinked poly(acrylic acid) hollow spheres that undergo a pH-induced swelling transition.

Stimuli responsive Nanocapsules

- The carboxylic acid groups of the polymer particles of the systems dissociate increasingly with the rise in pH.
- Generating a high negative charge density along the polymer backbone .
- These containers can retain encapsulated material at low pH and released it in “one shot” at high pH.
- Example : pH-responsive microparticles have been proposed for the oral delivery of insulin.
- The insulin-containing particles retain the substance at low pH in the stomach until they reach a higher pH in the intestine.

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- Delivery system consists of insulin containing micro particles of crosslinked copolymers of poly(methacrylic acid)-graft-poly(ethylene glycol).
- Reversible formation of interpolymer complexes stabilized by hydrogen bonding between the carboxylic acid protons and the ether groups on the grafted chains led for pH sensitivity.

Stimuli responsive Nanocapsules

Nanoparticle Films

- The formation of nanoparticle layers on a solid support in a “bottom up” approach.
- These thin films are formed by surface-modified nanoparticles.
- The attractive electrostatic interactions between charged nanoparticles and functionalized surfaces are frequently exploited.
- variations in the pH of a solution can be used to control the degree of ionization of the particle surfaces.

Nanoparticle Films

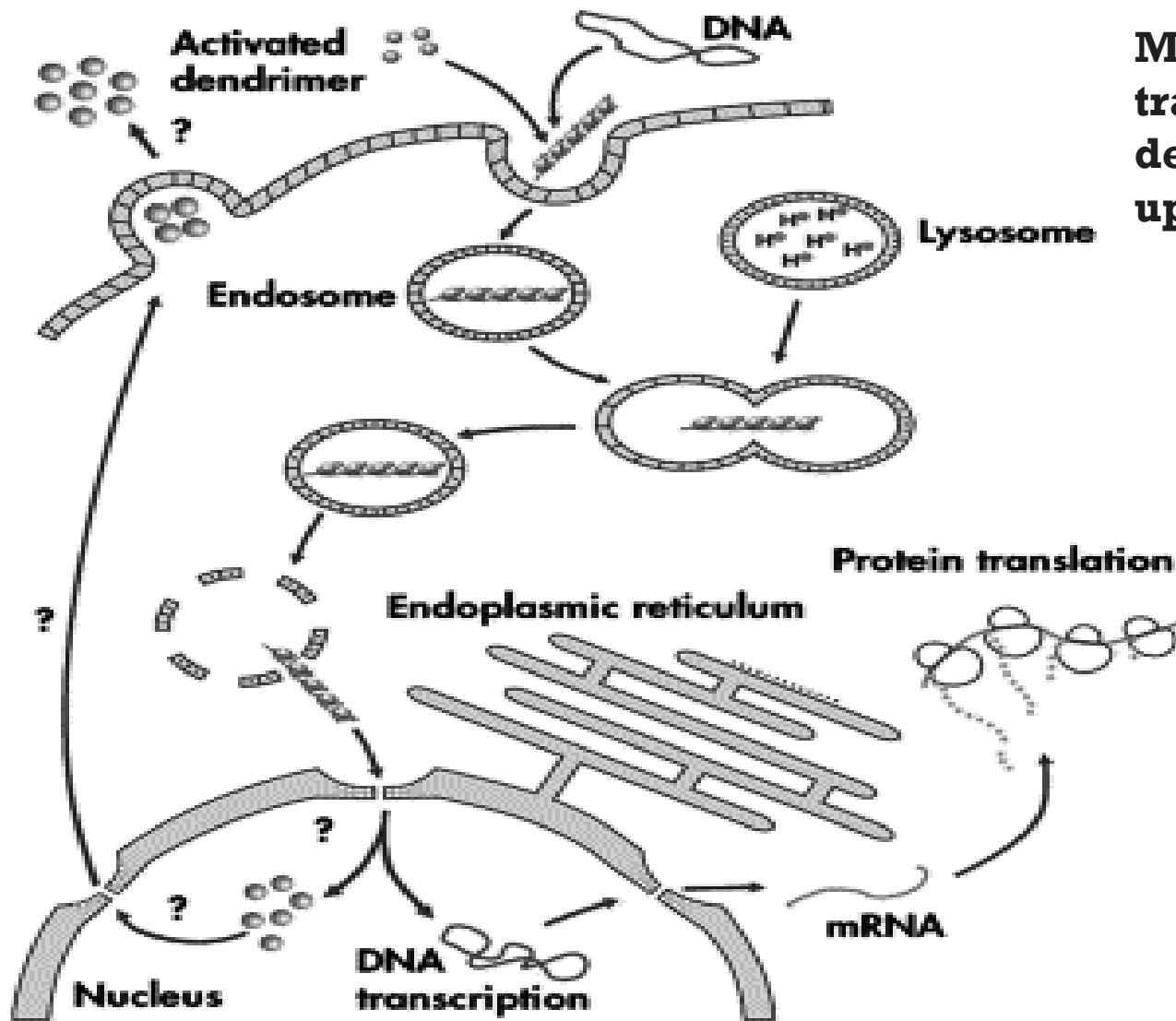
- Modulation of the electrostatic interactions between nanoparticles and the immobilizing surface.
- Examples:
 - a) Dendrimers : The large number of end groups at the periphery of a dendrimer and the relative ease of their tailoring leads to a plethora of pathways for surface recognition.
 - b) SCKs: surface-functionalized to promote cell binding via conjugation between the SCK nanoparticles and a biologically active peptide sequence.

Biomaterials and Gene Therapy

- Field of biomaterials focuses on the design of “intelligent” materials.
- that is, it can respond to their surrounding environment to improve their integration and function.
- Example: polymer nanoreactors can be used for the incorporation and controlled release of polypeptide growth factors that are inherent to biological function regulation (e. g., tissue regeneration).
- An alternative approach to viral gene delivery: complexation of DNA with cationic lipids, polycationic polymers, and dendrimers

Biomaterials and Gene Therapy

- Both liposomes and biocompatible block copolymer nanocontainers can be used.
- enables the genetic material to be protected against the action of endonucleases.
- due to their limited blood clearance and drainage into the lymphatic system (in case of tissue injection).
- Further, block copolymer chemistry would allow the preparation of nanocontainers with the potential to encapsulate large quantities of guest molecules within their central cavity,



Model mechanism of DNA transfection using active dendrimer mediated uptake.

Outlook

The principle of combining the high diversity of polymer chemistry with the functionality of natural proteins and peptides will have many future applications in areas such as :

- a) drug delivery,
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Outlook

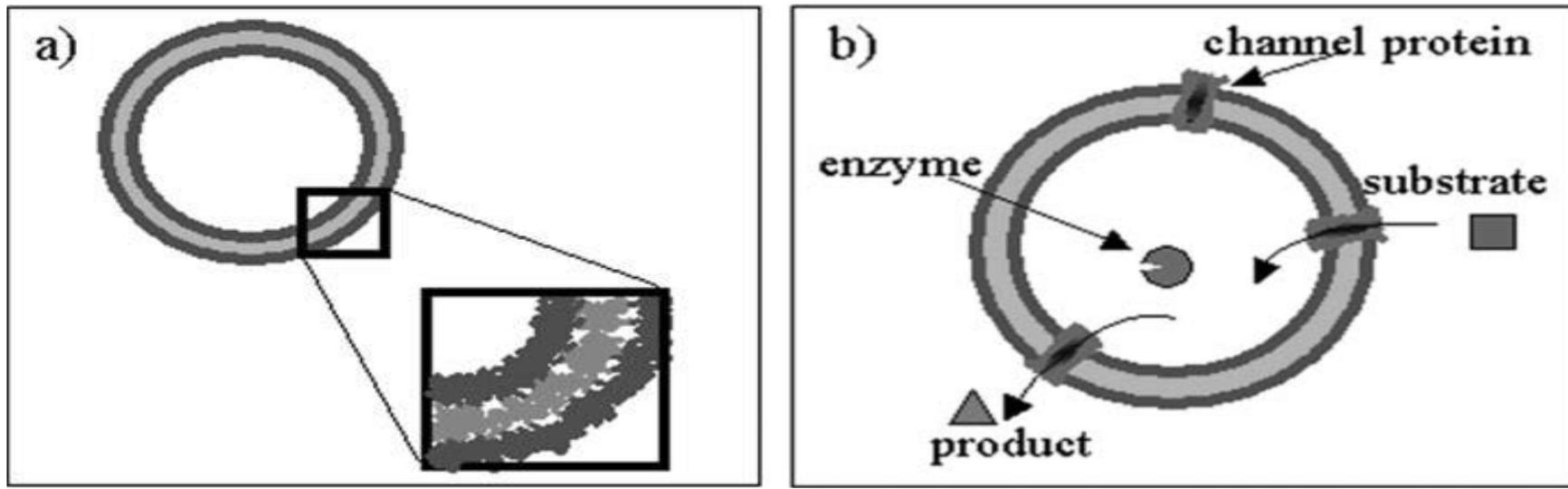
- The possibility of incorporating biological functions into these synthetic structures.
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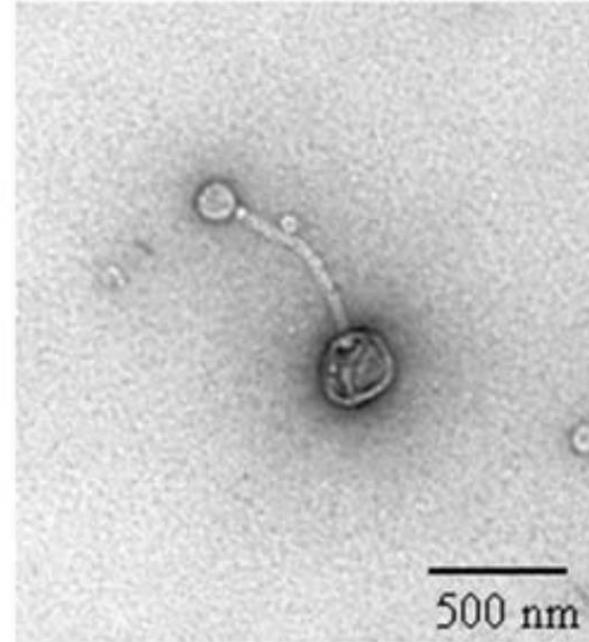
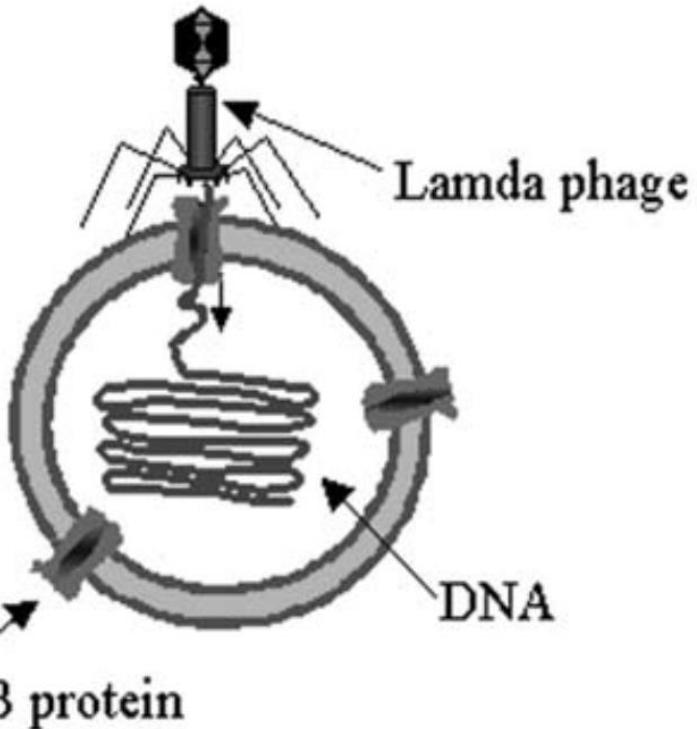
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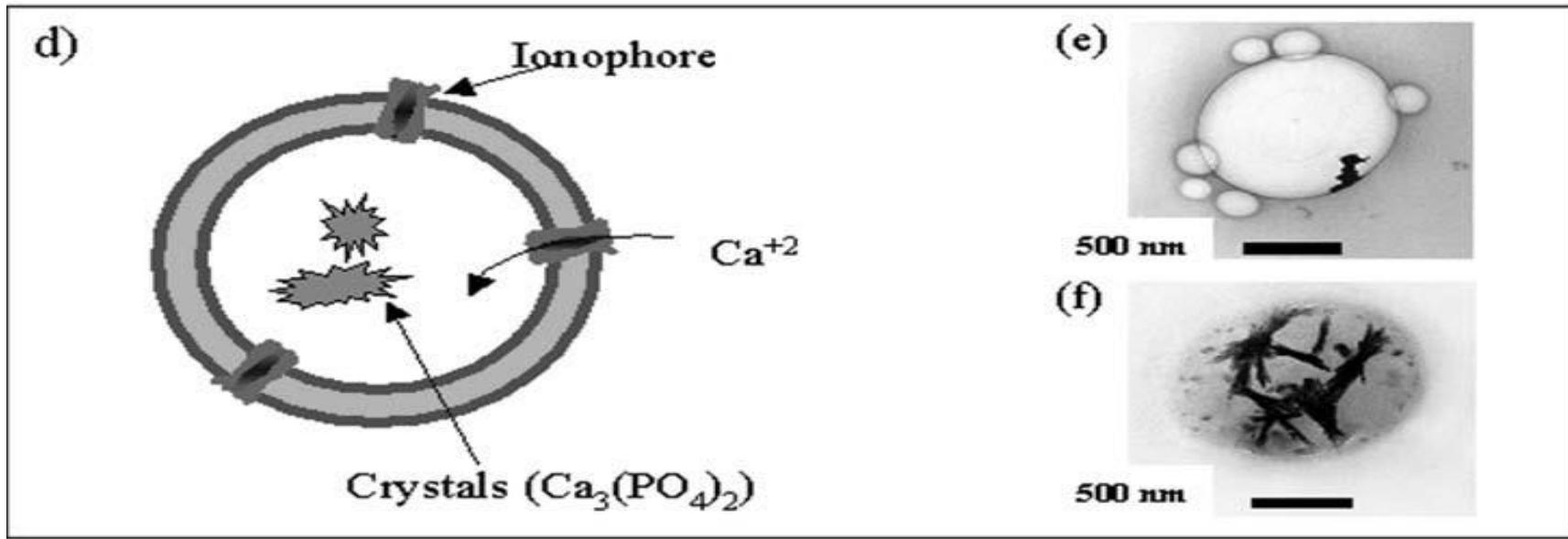
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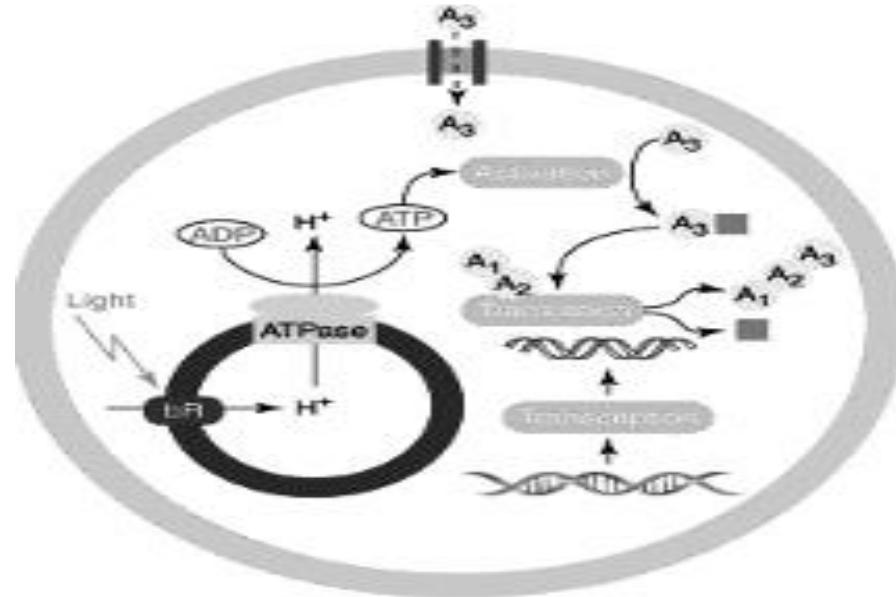
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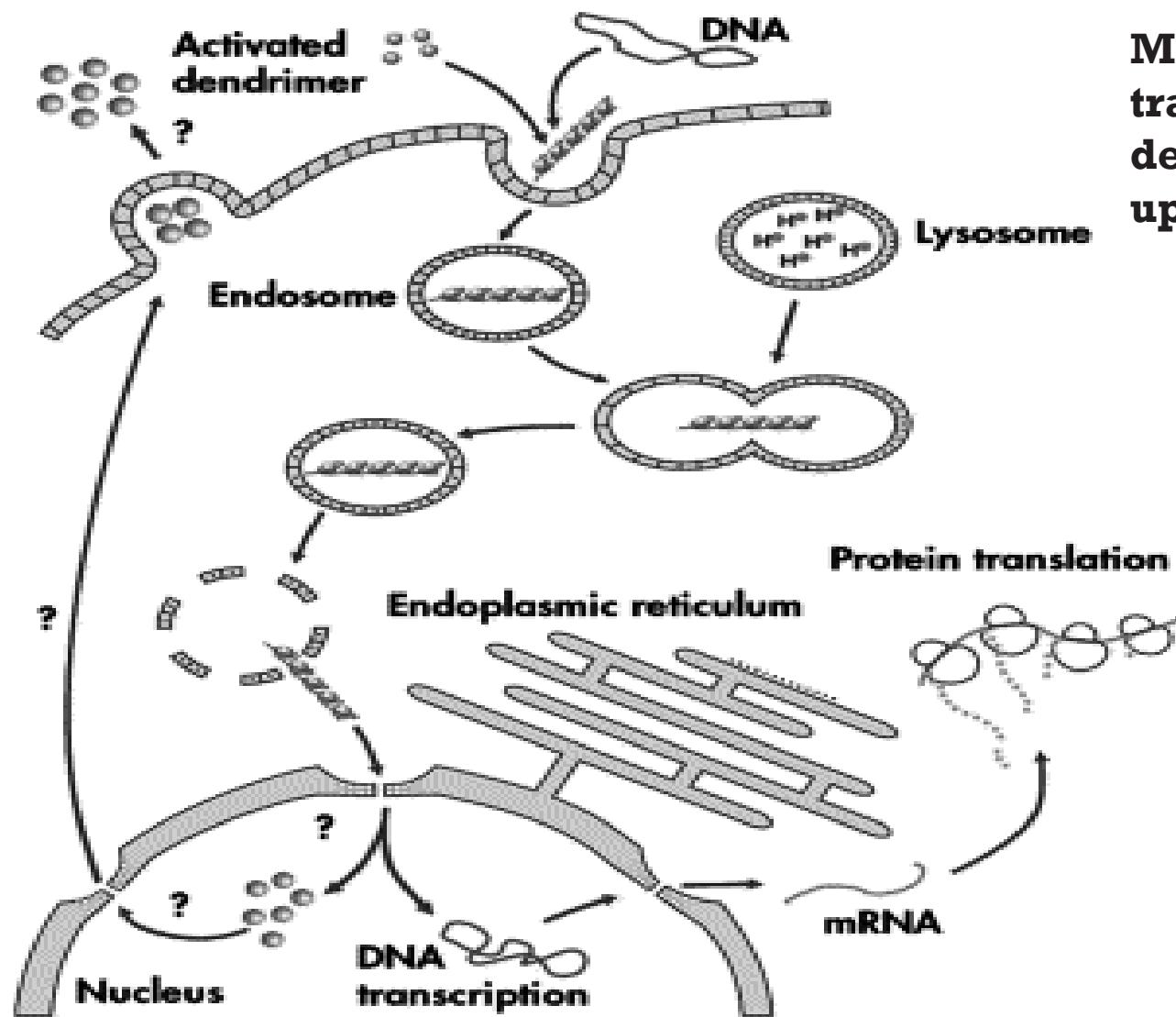
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A dark blue background filled with numerous small, glowing 3D cubes of varying sizes, creating a sense of depth and motion.

Molecular Lego

Design for Molecular
Actuators

Molecular Lego

- CREATING NEW NANOSTRUCTURES: Proteins are made practically with a collection of building-block of molecules developed to Join together and form rigid structures whose overall shapes are completely preplanned by the designer, like a model made a tiny, oddly shaped Lego bricks.

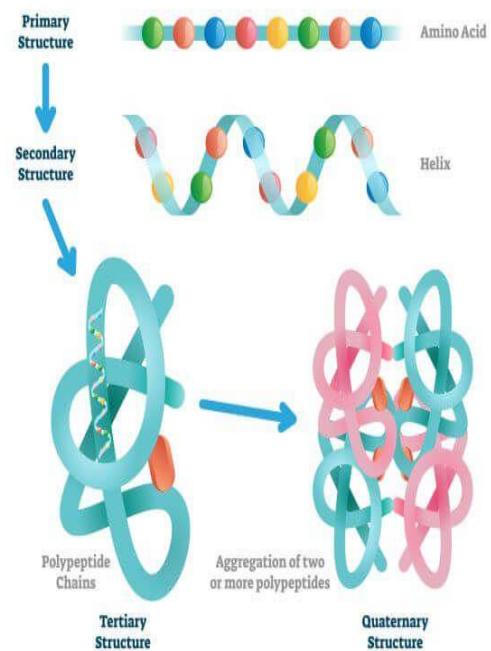
Molecular Lego

- Proteins are the fundamental nanomachines of life and serves the basis to create a nanomachinery.
- Every protein generally starts as a simple, linear chain assembled from a specific sequence of amino acids drawn from a repertoire of just 20 amino acids.
- The properties of a protein and what functions it can play out depend on its shape.

Molecular Lego

- The chain of amino acids is put together in the cell where it forms an intricate tangle of helices and other structures through a complex process called **protein folding**.
- The sequence of amino acids determines the final shape, but predicting what shape a particular sequence will take on is one of the most significant unsolved challenges of science and engineering (the “**protein folding problem**”).

PROTEIN STRUCTURE



Applications of Molecular Lego

- A way to produce large molecules with programmable shapes and the computer software required to design them was developed.
- To create sensors: large molecules that change shape and color when they bind to **particular target molecules such as glucose, toxins or chemical warfare agent**.
- The binding event triggers the sensor molecule to swing two fluorescent groups together that alter its color, thereby signaling that the target is present in the sample.

Molecular Lego

- Technique to create long, hinged molecules that open and close in response to an external signal is a step toward the creation of molecular actuators, molecular valves and computer memories.

Molecular Lego

- Example 4HB1, an artificial protein.
- An artificial gene was first assembled and then inserted into a bacteria, which "expressed" it - that is, made the protein encoded by the gene's DNA.
- After crystallization the structure was visualised to find it had the configuration for which it was designed.
- yet it was determined that the protein folding steps may be more complex than it appears to be.
- better way to create custom-designed nanomachinery would be to construct them from a limited set of modular building blocks that did not attain their shape via the folding process of proteins.

Molecular Lego

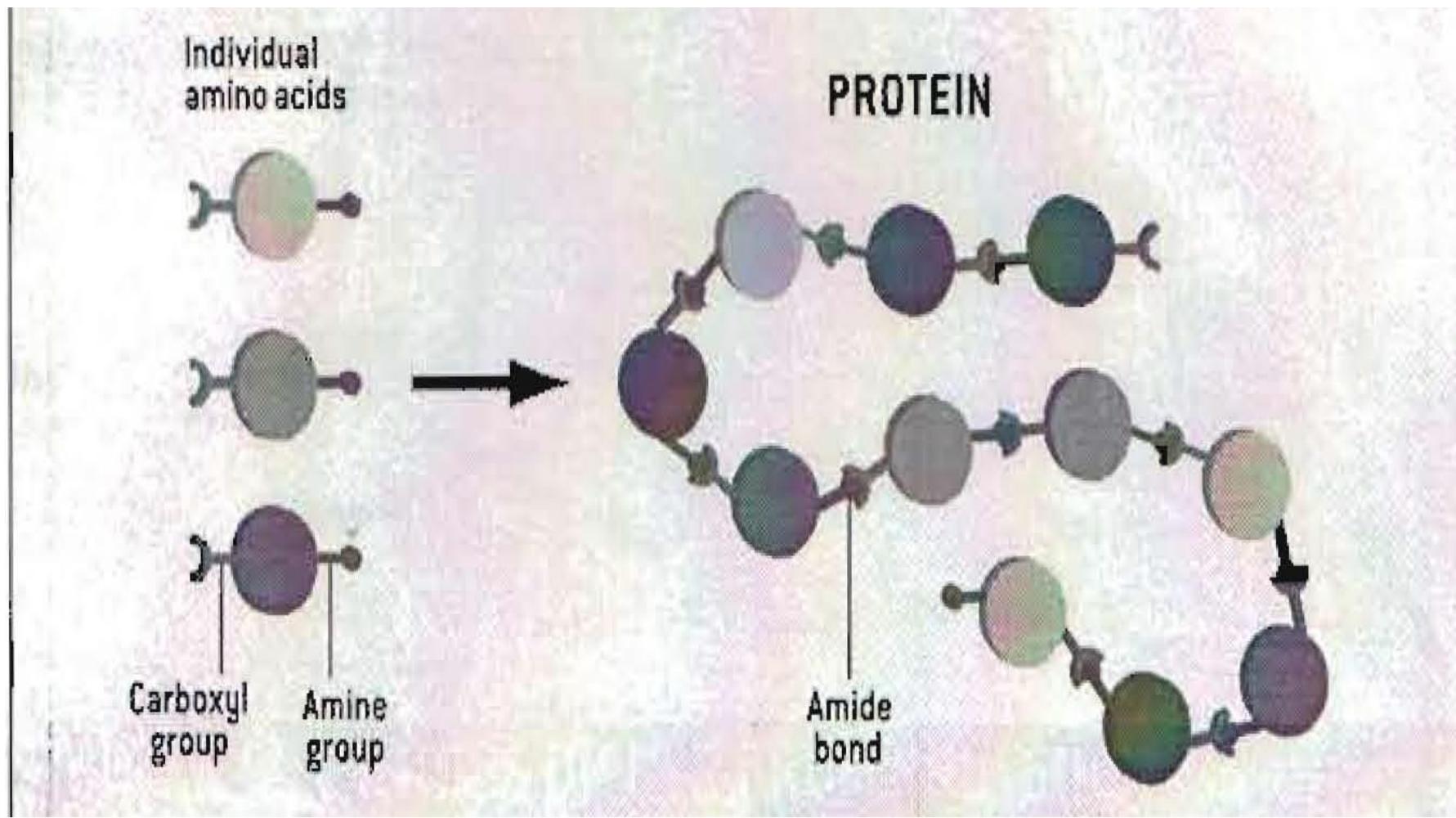
- 1995, Brent Iverson of the University of Texas at Austin had developed building blocks that could be chained together into short polymers called **oligomers**.
- These **oligomers** then self-assembled into pleated structures as electron-rich donor groups pulled on electron-deficient acceptor groups in the structure.
- Sam Gellman of the University of Wisconsin Madison and Dieter Seebach of the Swiss Federal Institute of Technology in Zurich developed synthetic molecules called **beta-peptides**, which are flexible chains of beta-amino acids.
- These short beta-peptides fold into twisted helices.

Molecular Lego

- Beta-amino acids are molecules that are mostly not naturally occurring and whose general structure is slightly different from that of regular amino acids (alpha-amino acids) .
- Natural proteins and these new molecules involve chains of molecules connected by single bonds that leave the structure with a lot of freedom to bend at locations all along its length.

Molecular Lego

- The way one of these molecules bends in acquiring its final shape depends on the complex interplay of attractive and repulsive forces arising when different building block all along the chain are brought closer together.
- To eliminate the usual folding process altogether in order to gain more control over the shape of the final product.



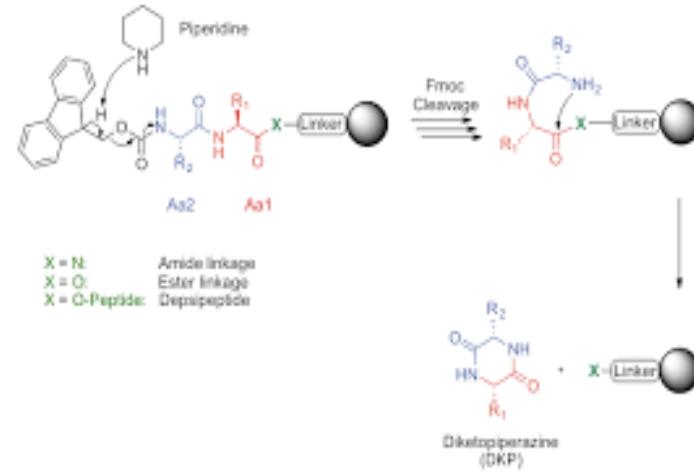
Molecular Lego

- Thus, rigid building blocks that could be attached to one another through *pairs* of bonds to create rigid, ladder like macromolecules were invented.
- 1987 J. Fraser Stoddart at the University of Sheffield in England, introduced the concept of a “**molecular Lego Set**” by creating molecular belts and collars from building blocks.
- These novel molecular building blocks called **Bis- amino acids**.
- These can be strung together to form protein like structures that have rigid, readily predictable and designable shapes.

Molecular Lego

- Potential applications of these **Bis- Peptides** include medicines, enzymes for catalysing useful, reactions, chemical sensors, nanoscale valves, computer storage devices.
- Chemical structure called a **diketopiperazine** forms when six atoms join into a ring containing two amide bonds.

Diketopiperazine Formation in Basic Conditions



Molecular Lego

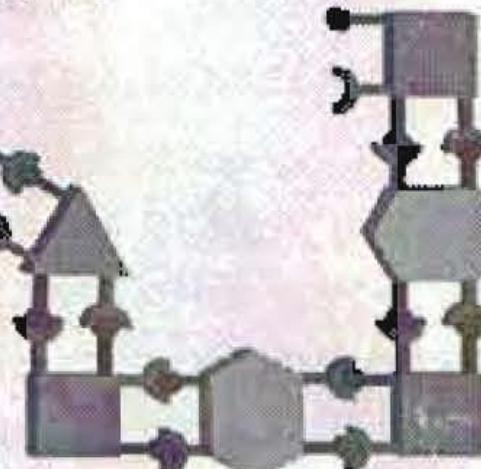
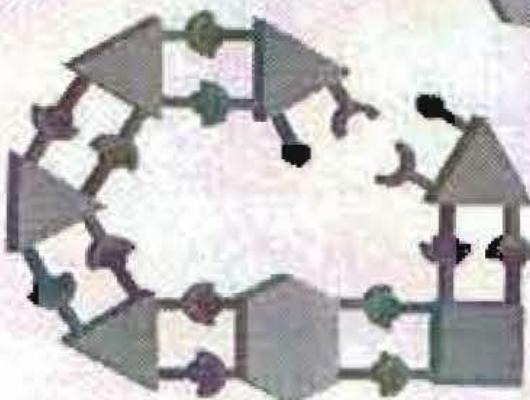
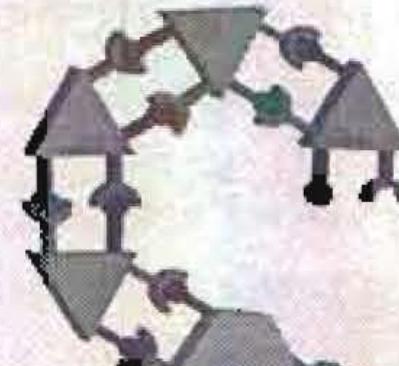
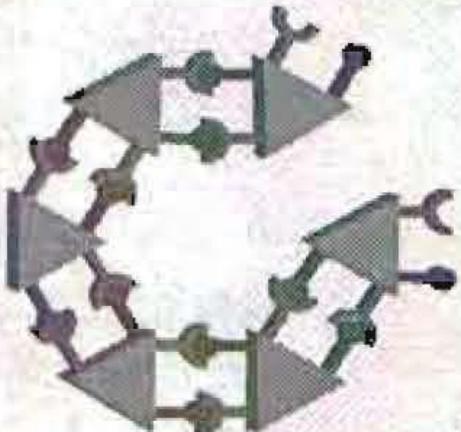
- Each monomer would consist of a rigid molecule of mostly carbon atoms with two amino acid groups integrated into it.
- And the amines and carboxyls of both amino acids would be available for bonding to other monomers.
- Two monomers would join by having an amino acid group on each one reacting together to form a diketopiperazine ring.

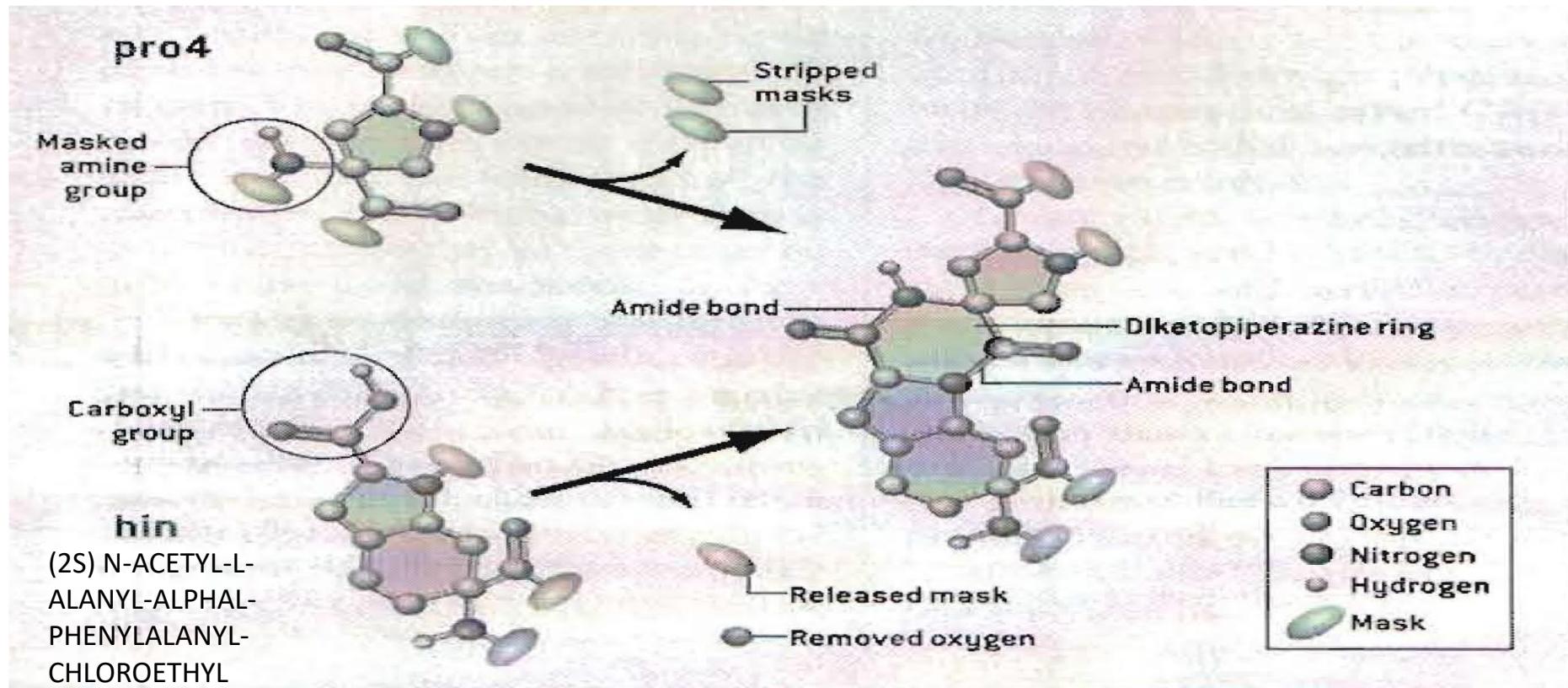
Molecular Lego

- The structure was called bis-amino acid ("bis" meaning "twice") because each one contains two amino acids.
- And just as chains of amino acids are called peptides, they were named "bis-peptides."

BIS-PEPTIDES

Individual
bis-amino acids

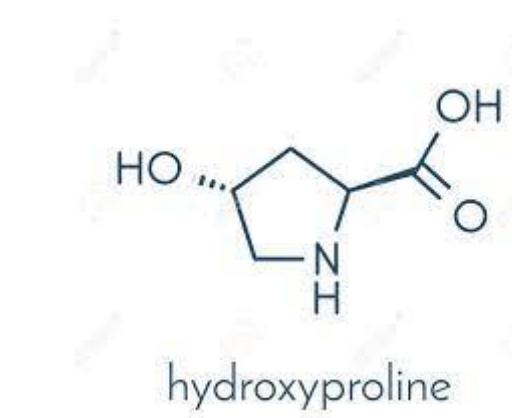




In practice, chemists synthesize bis-amino acids with protective groups, or masks, to prevent bonds from forming among them indiscriminately. Using a series of steps (*not depicted here*), the chemists link two monomers—such as pro4 and hin, whose chemical structures are shown at the left—by inducing what is called a **diketopiperazine ring** [green] to form between them. The rigidity of this ring and of the other carbon rings within the bis-amino acids ensures the stiffness and predictable shape of the resulting chains. [Some hydrogen atoms and details of the protective groups have been omitted for clarity.]

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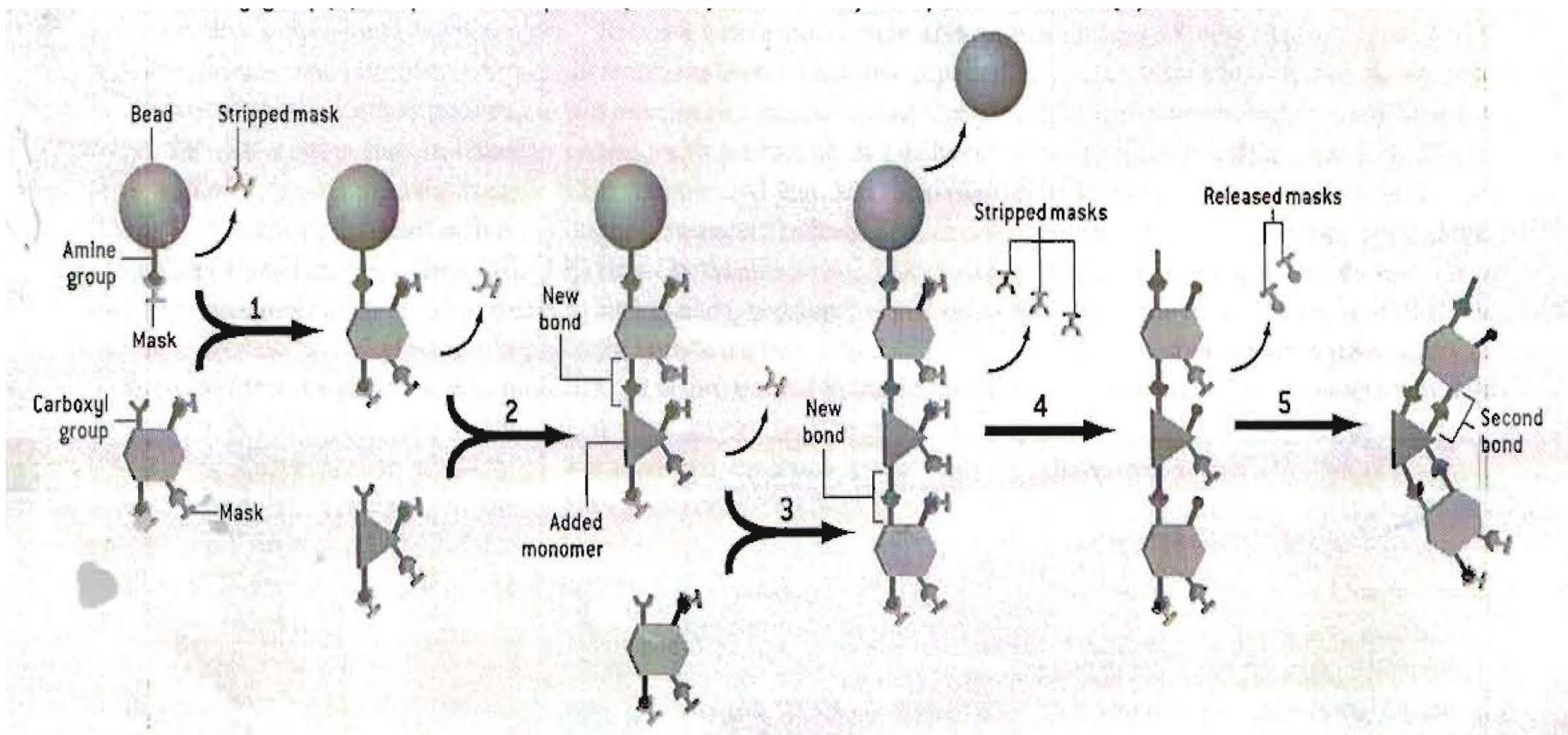
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HOW BIS-PEPTIDES ARE MADE

- (4) Then the bead is stripped away, as are the protective groups on the unbonded amine.
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Making of Bis-Peptide.



Programming Shapes

- The Bis- Amino Acids that make up the bis-peptides join together like strangely shaped Lego bricks.
- In particular, each bis-amino acid behaves like a brick whose top surface of studs is tilted and twisted relative to its bottom surface of holes.

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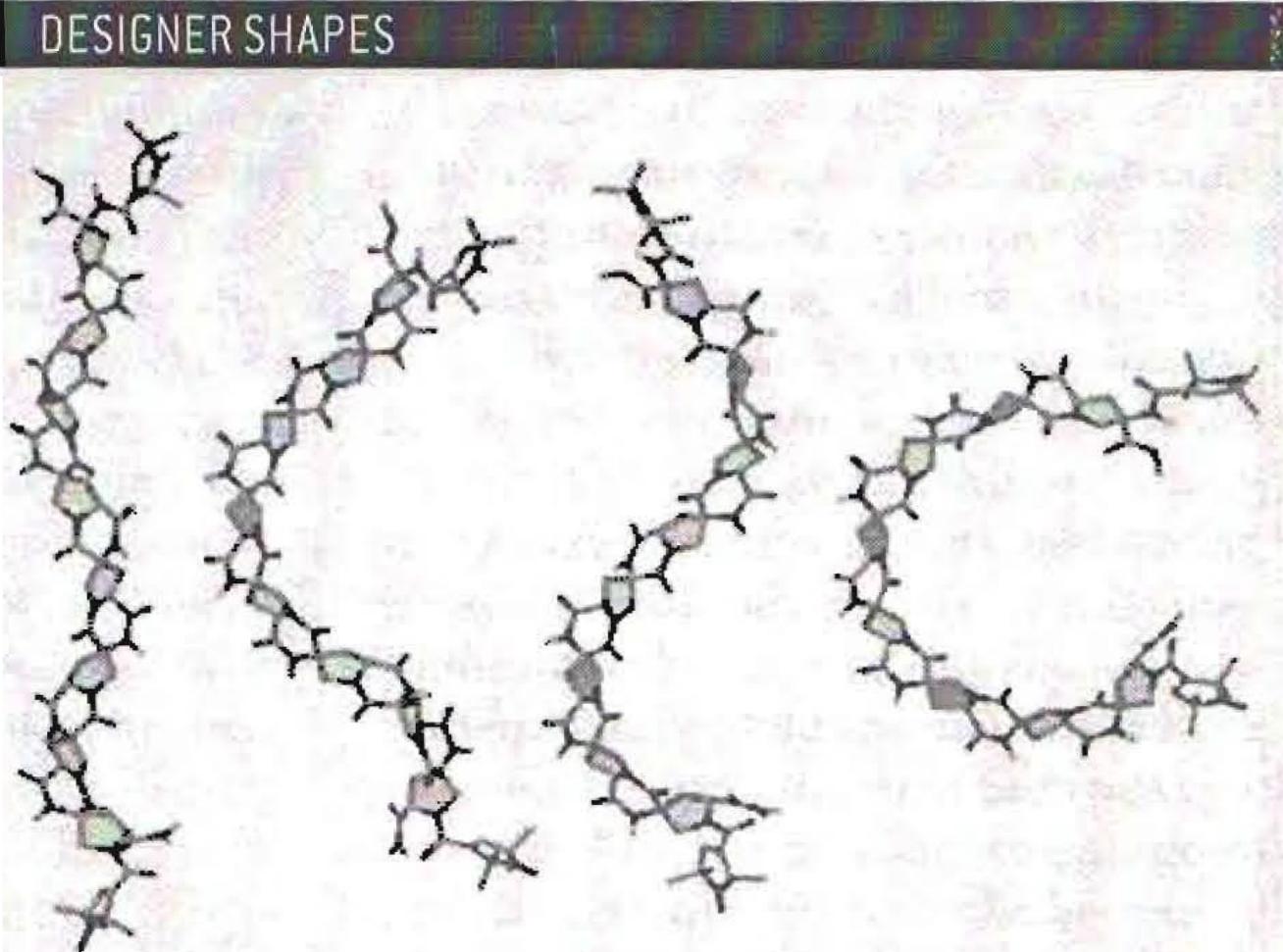
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The shapes of the molecules can vary from nearly straight rods to tight crescents with the insertion of the right monomers.



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- Macromolecule that would bind tightly to the cholera toxin protein (Ctx).
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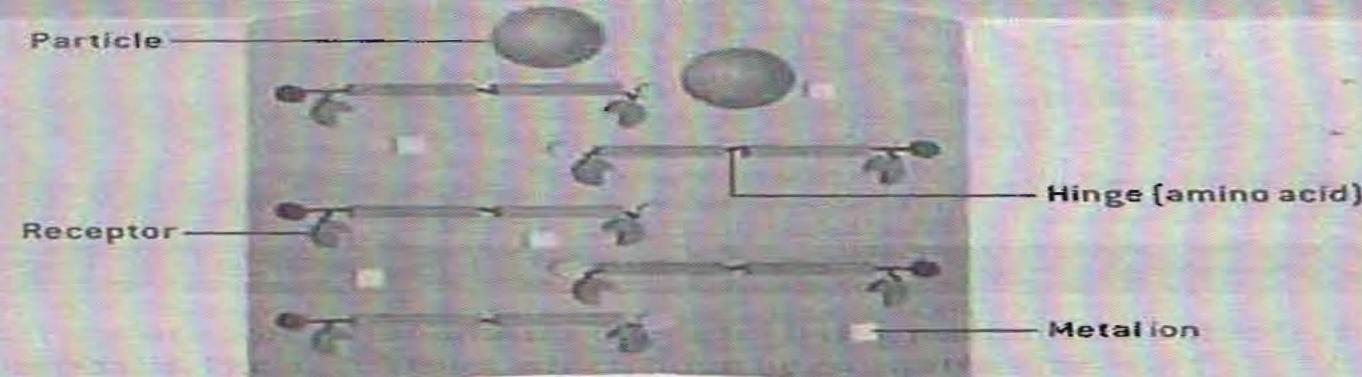
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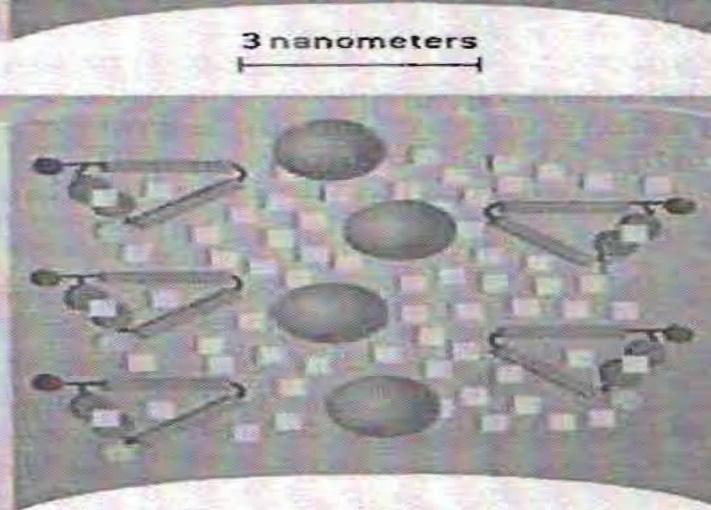
Development of Molecular actuators

- An actuator is a device that responds to a signal by producing motion.
- **Development of Molecular actuators** in which two rods are joined by a hinge.
- Rod-hinge-rod actuators were designed to be open normally and to fold over, or close, when groups on the outer ends of the rods bind a metal or a small molecule.
- The rods were of four building blocks long, the hinge of an ordinary amino acid, and a metal that triggered the opening and closing.
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NANOSCALE VALVES
CLOSED CHANNEL



OPEN CHANNEL



Developing Applications

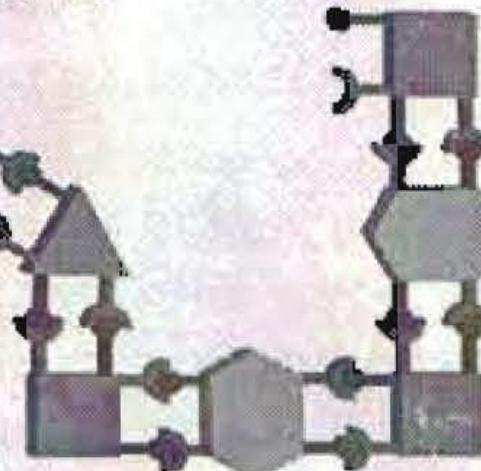
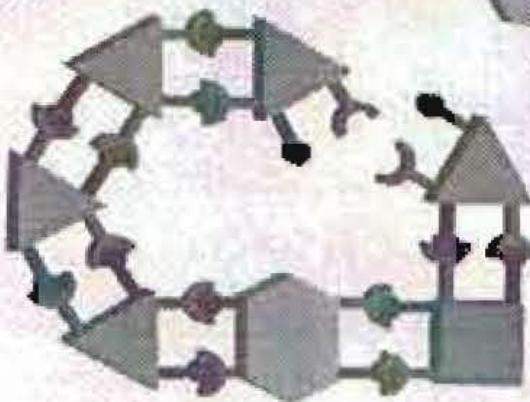
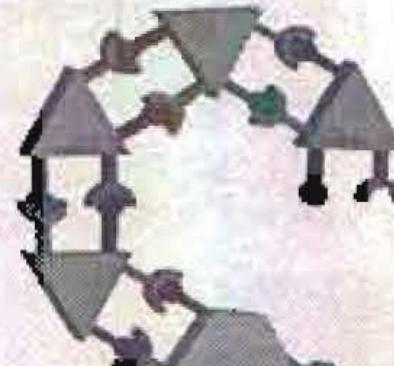
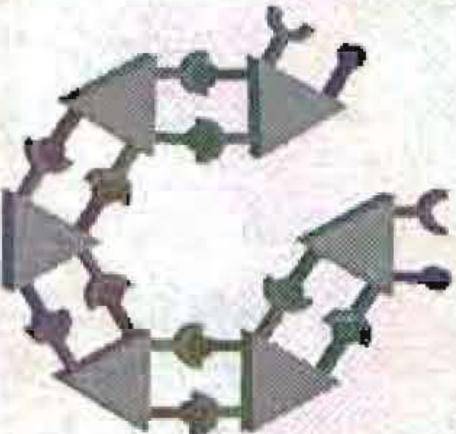
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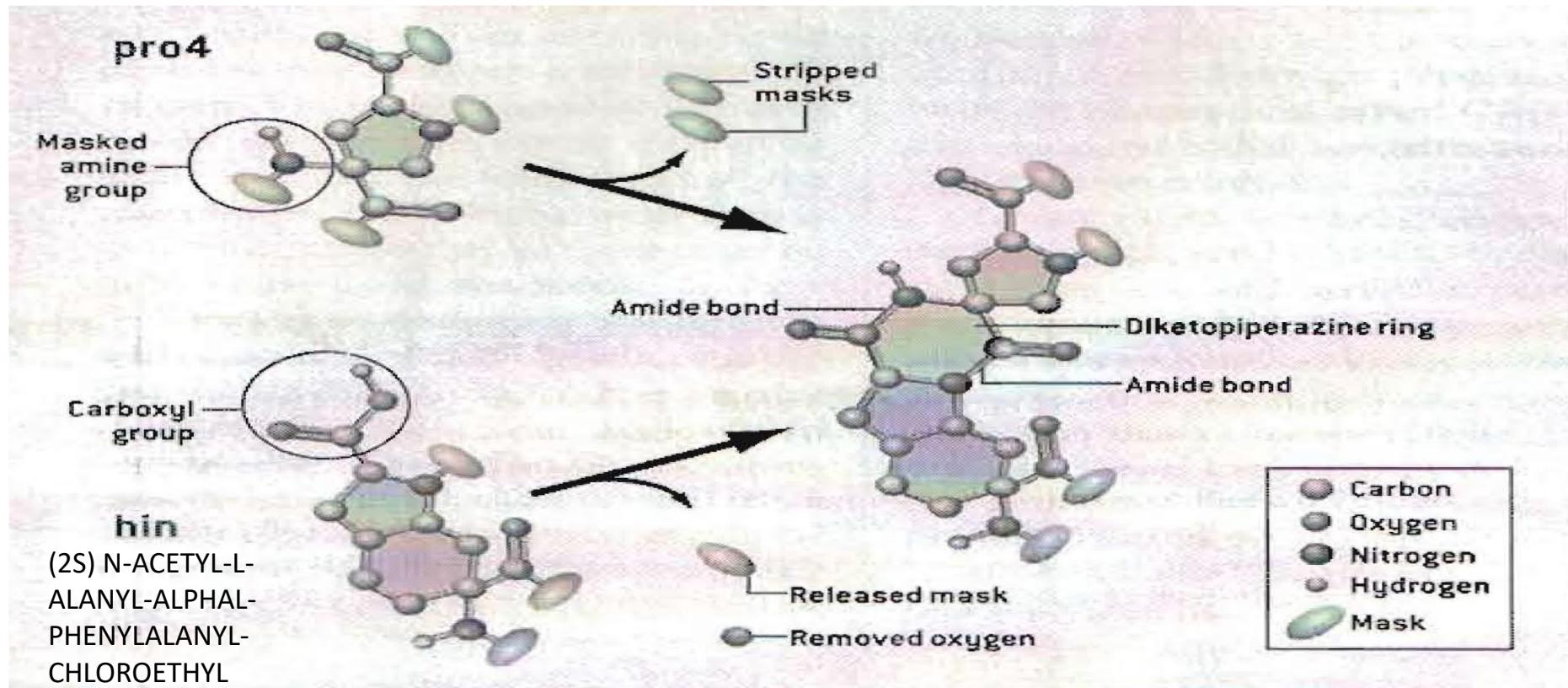
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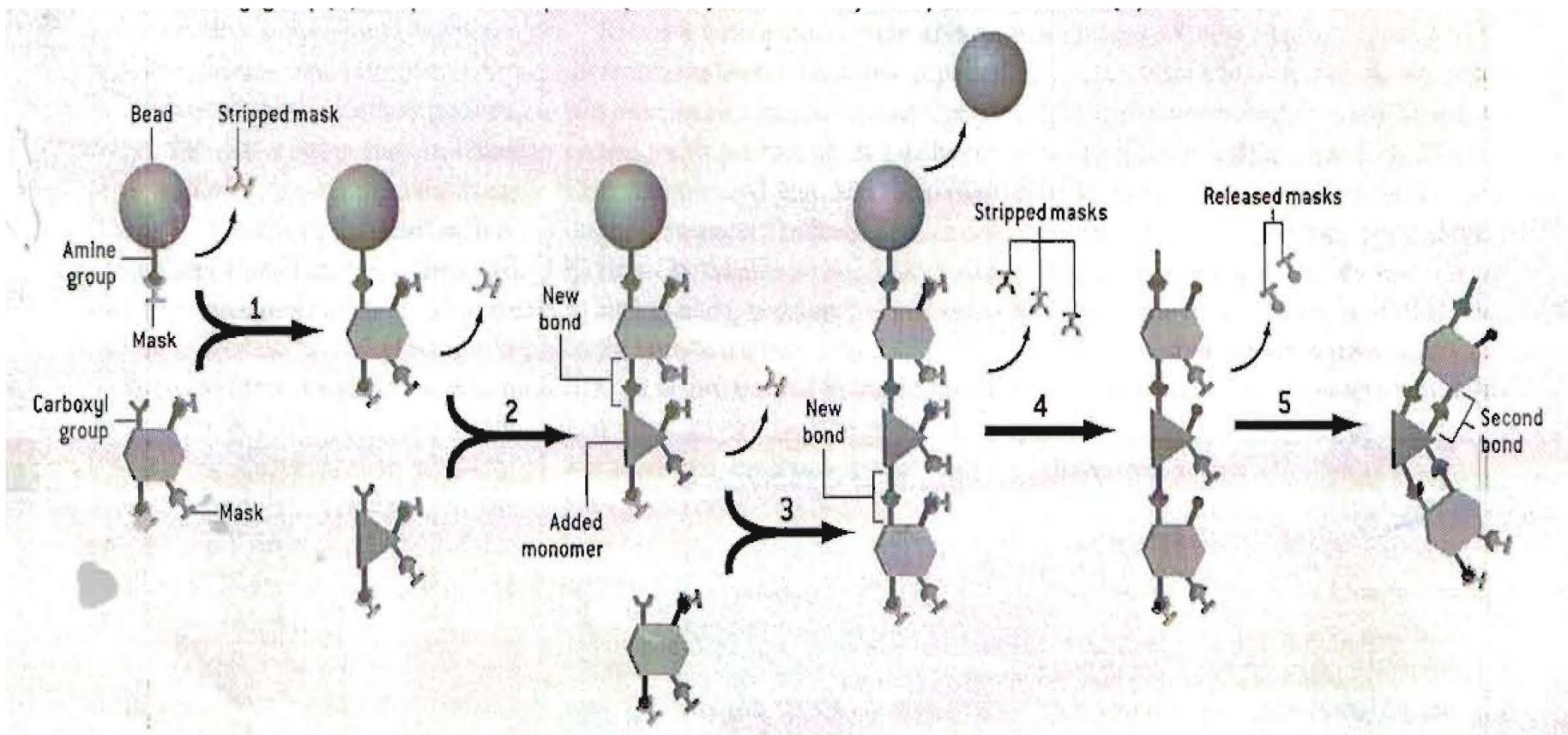
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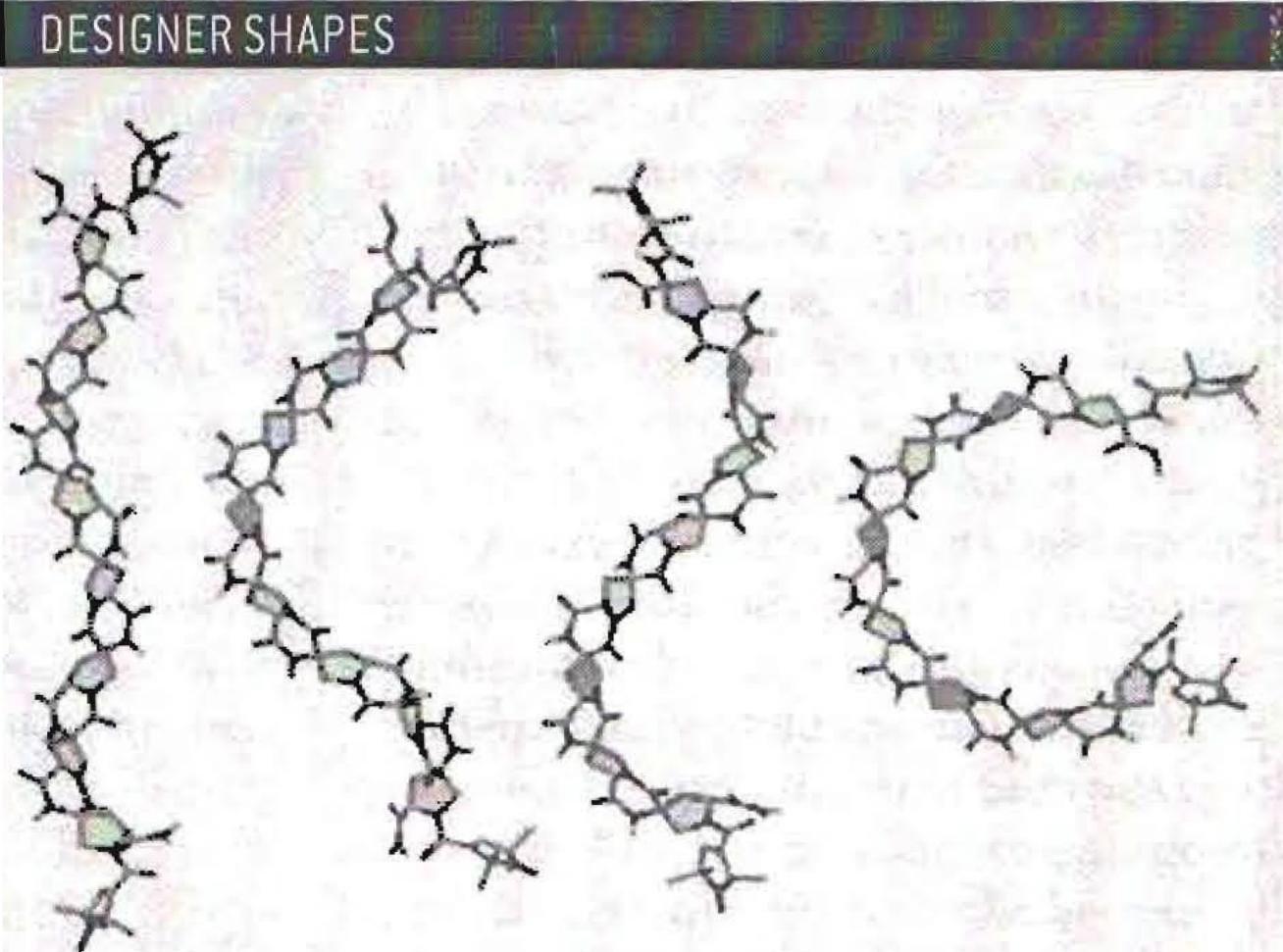
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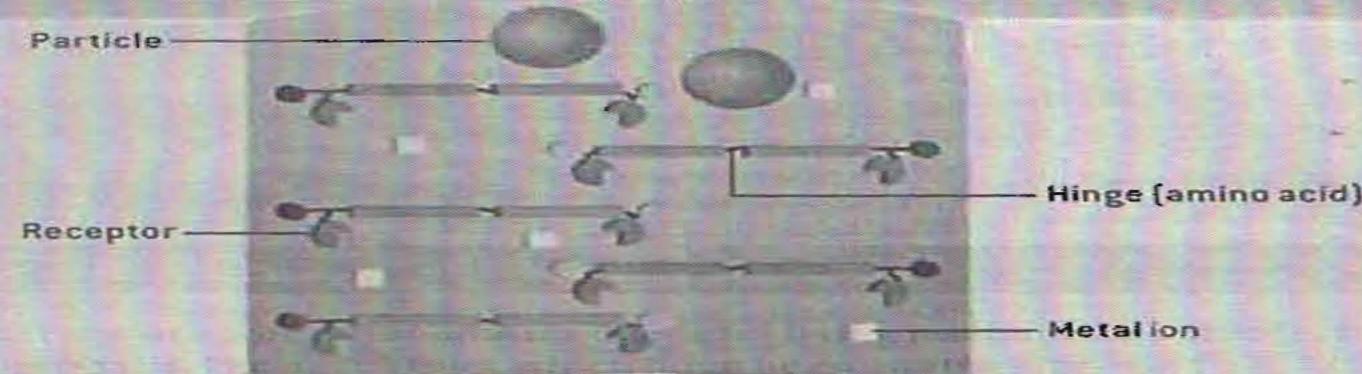
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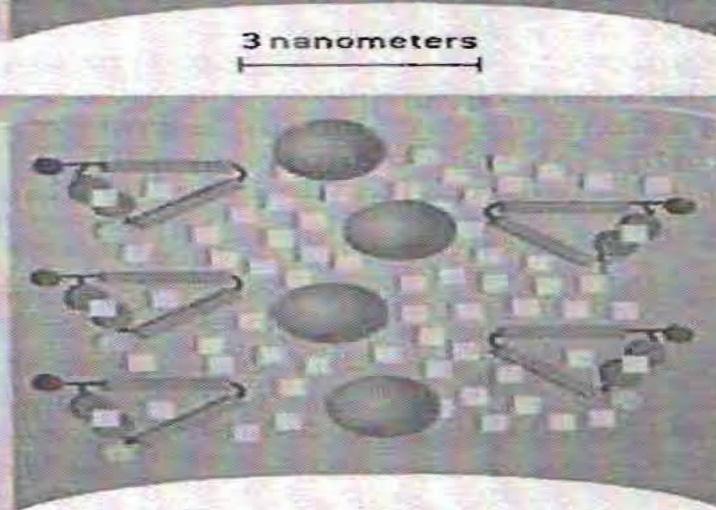
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Ion channels as molecular switches

(Patch clamp technique)

Bionanotechnology
BBL 747

Introduction

- The **transmission of signals within and between cells is mediated by ion channels, pore-forming proteins embedded in the plasma membranes of nearly all cells.**
- In sensory organs the channels translate physical or chemical stimuli into electrical signals for the nervous system.
- Even cells not connected to the central nervous system, such as those in the blood, immune system, liver and other organs, use ion channels for signaling processes.
- Since the 1950s biologists have been able to study the electric currents arising from these ion fluxes at a macroscopic level.
- Only since the 1970s, however, could they **examine the individual ion channels themselves.**
- The **patch clamp technique**, for which the Nobel Prize for Physiology or Medicine was awarded (1991)

Transport across Cell Membrane

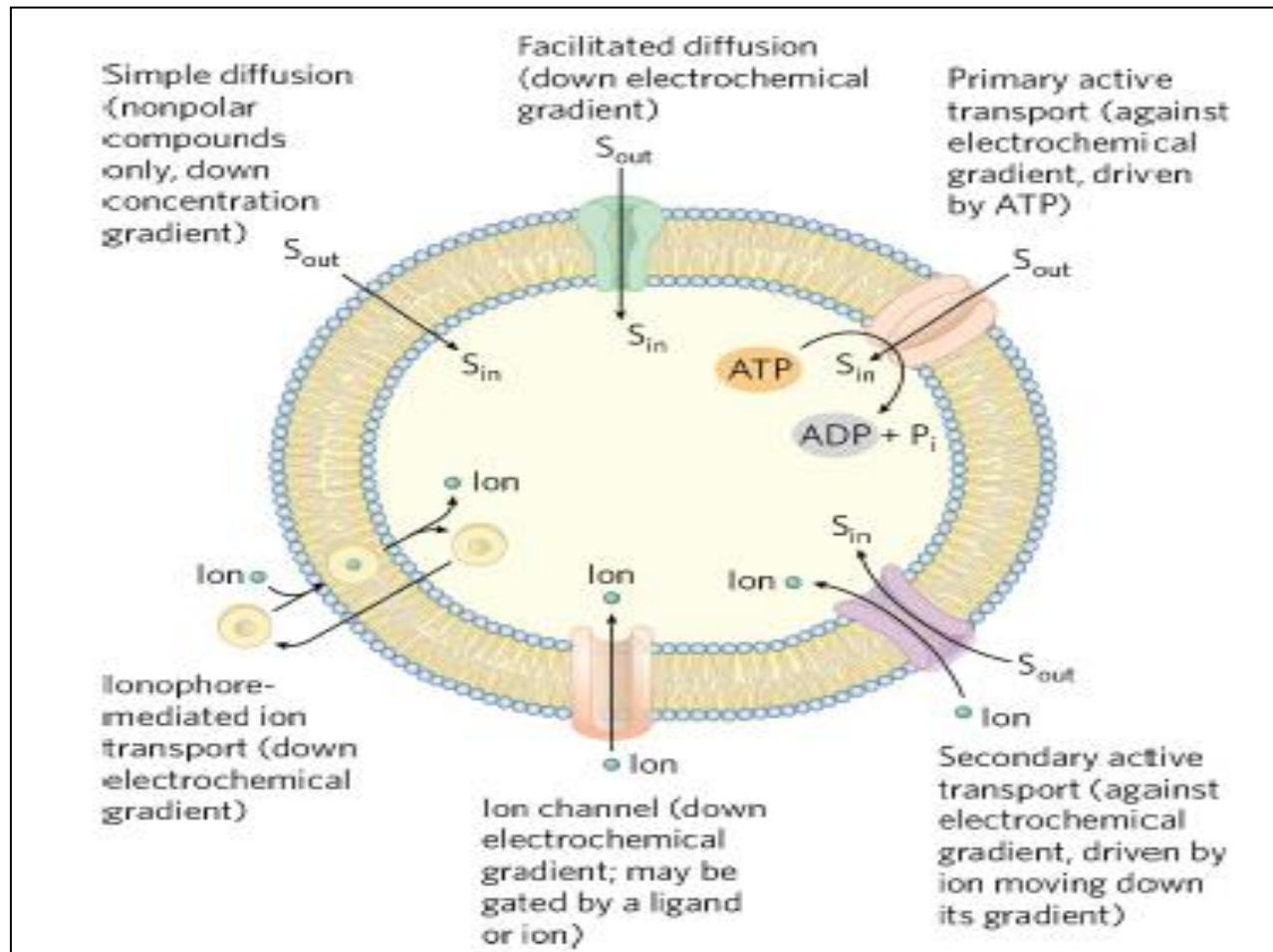


FIGURE: Summary of transporter types. Some types (ionophores, ion channels, and passive transporters) simply speed transmembrane movement of solutes down their electrochemical gradients, whereas others (active transporters) can pump solutes against a gradient, using ATP or a gradient of a second solute to provide the energy.

Patch clamp technique

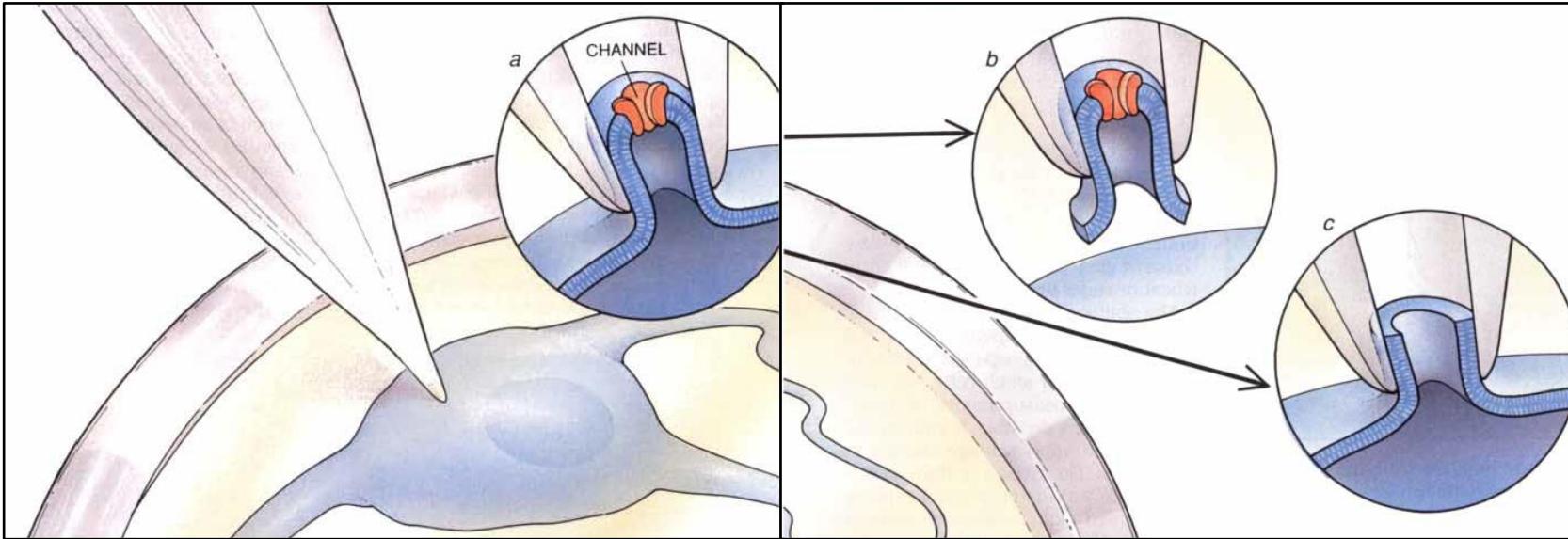
- Tight sealing a pipette against the surface of a neuron enables researchers to study the ion channels in the outer plasma membrane.
- The pipette, only 1/25,000 the diameter of a human hair, physically and electrically isolates the trapped channels.
- This technique, known as patch clamping, can record the opening and closing of ion channels.
- The technique is being used to discover the details of signaling networks within cells.



Patch clamp technique

- Although its development and refinement took several years, the technique is fundamentally simple: **a thin glass pipette of the proper shape is tightly sealed against a cell membrane, thereby isolating a small patch of the membrane and the ion channels it contains.**
- These **channels can then be chemically or electrically manipulated and their properties deduced.**
- A researcher can even remove a patch of membrane from a cell or carefully open a window into a living cell to alter its cytoplasmic constituents.
- In all these various applications the patch clamp technique makes it possible to probe how ion channels affect membrane voltage and cell processes such as secretion and contraction.

Three Forms of Patch Clamping



- By pressing a patch pipette against the enzymatically cleaned surface of a cell and applying gentle suction, researchers can place a giga ohm seal around a small patch of the cell membrane and the ion channels it contains. An experimenter can then apply various stimuli from within the pipette and measure the behavior 'Of the trapped channels.
- Alternatively, the experimenter may detach the membrane patch from the cell, thereby exposing the cytoplasmic mouth of the channels
- If the membrane patch can be ruptured without breaking the giga-ohm seal, the experimenter can alter the constituents of the living cell's cytoplasm.

Background

- **Artificial lipid membranes, which in their pure form are electrical insulators.** Yet Ross C. Bean and his colleagues demonstrated that **if trace quantities of certain antibiotics or proteins were inserted into the membranes, they became electric conductors.**
- The **discrete changes in the current passing through the membranes suggested that the proteins created pore-like channels that opened and closed individually.**
- Charged ions could then traverse the membrane through the open channels.
- It became clear to many investigators that the electrical signals in neurons and other cells must be mediated by similar proteins in the lipid plasma membranes surrounding them.
- Alan L. Hodgkin and Andrew F. Huxley had previously invoked the concept of ion channels in their classic analysis of currents through nerve membranes, for which they received the Nobel Prize in 1963.
- If measurements at an appropriately fine resolution could be taken, a whole microcosm of signaling molecules would be found.

Background

- In 1972 Bernard Katz and Ricardo Miledi conducted a statistical analysis of voltage fluctuations at the neuromuscular junction (the synapse between a motor neuron and a muscle fiber).
- They concluded that the synaptic signals consisted of small electrical events of the same magnitude as those associated with the artificial channels.
- But Katz and Miledi could only infer the properties of the channels from their analysis, which depended on several assumptions.
- **No method was available for directly measuring the unit events constituting the synaptic signal.**
- The **background noise** associated with the standard techniques for measuring the electric current passing through a cell membrane was only one ten-billionth of an ampere, but it was 100 times greater than the current of a unit event and drowned out its signal.

Background

- The **available electronic components would have the resolution necessary for measurement only if a small patch of membrane could be insulated from the rest.**
- The approach was to place a glass micropipette onto the surface of enzymatically cleaned muscle fibers.
- The nonconductive glass pipette, isolated a few ion channels, thereby providing with a clear signal
- Unfortunately, obtaining a tight seal between a measuring glass pipette and a membrane was not easy.
- to put up with electrical leaks that connected the extracellular fluid with the interior of the pipette.
- Nevertheless, by carefully cleaning the cell surface and by optimizing the shape and size of the pipette, they succeeded in observing individual channel currents in response to acetylcholine, the transmitter (signal-inducing chemical) at the neuromuscular junction

Background

- It confirmed many previous inferences about the elementary currents through ion channels, in particular the assumption that they were pulse-like events with constant amplitudes and varying durations.
- **The low quality of the pipette-to membrane seal and the attendant background noise initially precluded from making detailed recordings of ion channels other than those found at the neuromuscular junction.**
- It was discovered that the **application of a slight suction through the pipette, together with some other procedural changes, could raise the resistance of the seal to more than a billion ohms-an improvement of several orders of magnitude.**
- By gently pulling, with the attached pipette, it was even possible to excise microscopic patches of the membrane for study in isolation.
- Thereafter, the recording of single ion channels became a high resolution technique.

Receptor Channels

- The most **detailed information on the role of ion channels in synaptic transmission has been obtained from experiments on the neuromuscular junction of skeletal muscle.**
- There the **presynaptic neurons release acetylcholine in discrete multimolecular packets called quanta.**
- Acetylcholine molecules bind transiently to acetylcholine receptor channels, specialized proteins in the postsynaptic membrane, and cause a current to flow across the end-plate membrane.
- This end-plate current is the sum of the elementary currents through hundreds of thousands of channels.
- To measure these elementary currents individually, one can press the tip of a patch pipette onto a muscle fiber at the end plate.
- This region of muscle surface contains the acetylcholine receptor channel.
- When a low concentration of acetylcholine is in the pipette solution, the current recorded with the pipette switches between two levels.

Receptor Channels

- At one level, effectively no current flows because all the ion channels in the membrane patch are shut.
- When one channel molecule flips to the open state, because of a voltage applied to the membrane, a current of about 2.5 picoamperes abruptly flows through it.
- After a variable period, the molecule flips back to the shut state, and the current switches off.
- The opening of the receptor channel is triggered by its binding to acetylcholine the unbinding of acetylcholine causes the channel to close again.
- The **randomness of the lengths of time for which the channel stays open and closed reflects the probabilistic nature of the interactions between the acetylcholine molecules and their receptors.**
- The amplitude of the current steps represents the capacity of the channel to transport ions, such as sodium or potassium in the case of the acetylcholine receptor channel.

Receptor Channels

- **By comparing the measured current amplitudes and duration distributions with the predictions of various hypotheses, an investigator can determine how ions interact with a channel molecule and how the interaction between a transmitter and its receptor controls the opening and closing of the channel.**
- In synapses of the central nervous system, amino acids such as glycine, gamma-aminobutyric acid (GABA) and L-glutamate are the most prominent signaling substances for rapid communication.
- **The pulse-like shape of the currents measured for channels that bind these transmitters indicates that they, too, open and close randomly.**
- The receptor channels may therefore work in essentially the same way as do the acetylcholine receptor channels at the end plate.
- Nevertheless, transmitter-gated channels in the central nervous system often show an additional complexity, in that some channels may be only partially open or closed and that different subtypes of the channels may occur in various brain regions.

Receptor Channels

- The transmission of information from the central nervous system to the neuromuscular junction needs to be extremely rapid.
- In the myelin-coated neuronal axons that carry those signals in vertebrates, conduction is not mediated by transmitter-gated channels like those we have already described.
- Instead, it is mediated by faster channels that respond to changes in membrane voltage—the difference in electrical potential between the inside and outside of the cell.
- Voltage-gated channels for sodium ions underlie the rapid rise of the action potential in neurons.
- Analyses of the elementary sodium currents indicate that the voltage-sensitive channels switch between two states and have a high probability of opening shortly after the beginning of a voltage change.
- The currents supporting the nerve impulse are generated by the superposition of tens of thousands of such elementary sodium currents.
- Voltage-gated channels for other ions, such as potassium and calcium, seem to operate in much the same way as sodium channels do and share many of the same structural features.

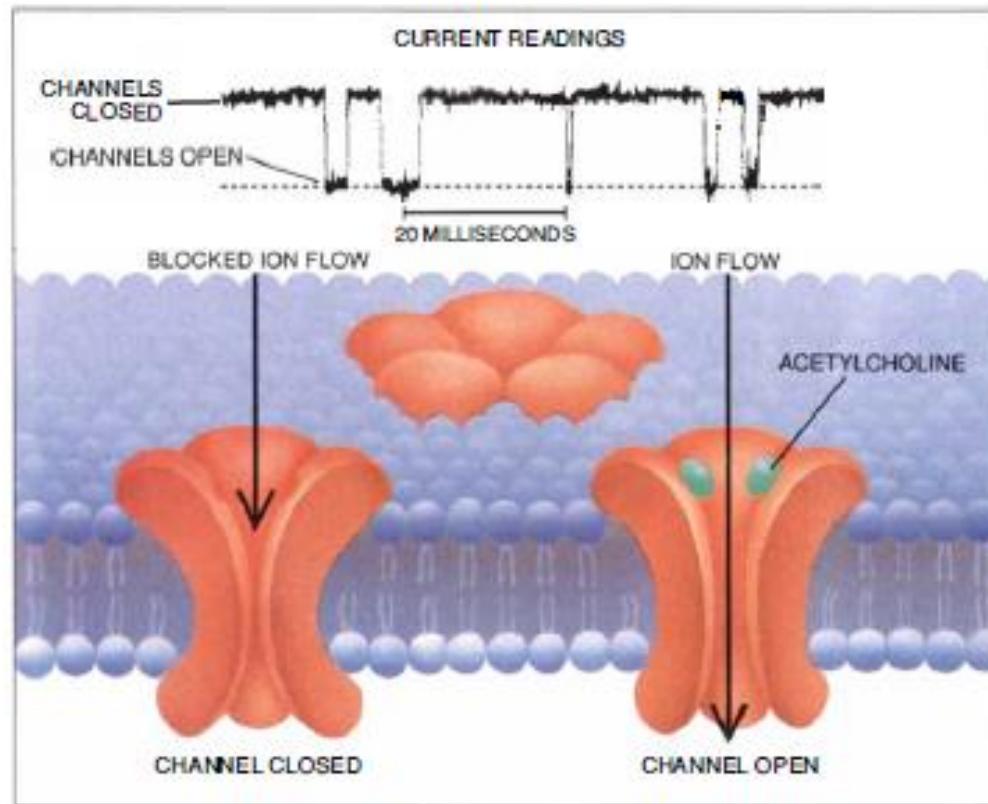
Receptor Channels

- Patch clamp investigations have revealed the dynamic molecular mechanisms of transmitter- and voltage-gated channels at a level of finer detail
- The **elementary event is not a single current pulse but a series of transient pulses separated by brief gaps.**
- When a receptor channel in the end plate of a neuromuscular junction binds with acetylcholine, for example, it opens and closes several times before the acetylcholine finally dissociates from it.
- The structure of these transitions for end-plate channels was investigated
- Using probability theory, the number of states an acetylcholine channel adopts during its succession of openings and closings and also the rates of the transitions from one state to another was estimated .
- **Each receptor has two binding sites for acetylcholine**, when both sites are occupied by acetylcholine molecules, the probability that the channel will open is close to 100 percent

Receptor channels

Figure:

- Receptor channels found at the neuromuscular end plate open in response to the transmitter acetylcholine.
- When no acetylcholine is present, effectively no current passes through a channel.
- When acetylcholine binds to the receptor, an elementary current of a few picoamperes flows.
- The measured durations of the currents and the intervals between them vary because the interaction of acetylcholine molecules with the receptors is governed by probability.



Heterogeneity of Membrane Channels

- **Membrane channels are apparently heterogeneous: in the same membrane patch, two or more classes of closely related channels often seem to be activated.**
- Pharmacologists have known for many years that different subtypes of receptors exist, but the sharper tools now available in the form of recombinant DNA techniques have shown that the diversity of subtypes is far greater than had been imagined
- For each type of transmitter- or voltage-gated channel, there are several versions or subtypes with different conductance or gating properties that form a channel family.
- **External stimuli and the developmental stage of an organism can alter the mosaic of channel subtypes found on cell membranes.**

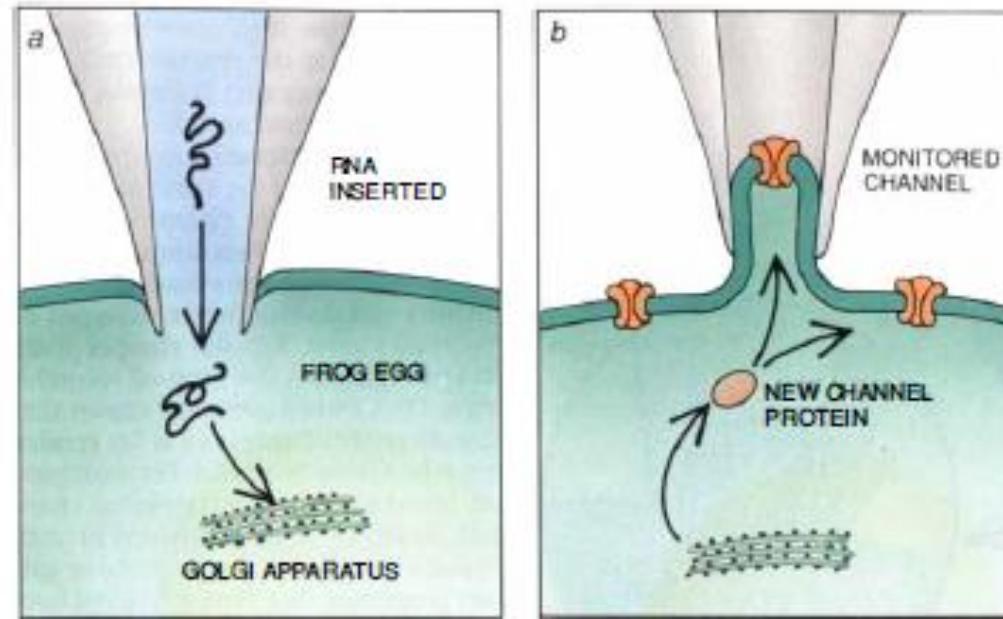
Genetic Studies

- A primary aim in the understanding of membrane channels is to relate their functional properties to their three-dimensional structures
- To attain this goal is **to locate and change critical sequences of amino acids in the channel protein** and then to **observe the effect of the alterations** on the channel's function.
- After the DNA encoding each subunit of the acetylcholine receptor had been cloned and sequenced, it became possible to perform such experiments.
- **Studies were performed on normal and genetically altered acetylcholine receptor channels in the membranes of host cells, such as eggs from the clawed frog Xenopus.**
- From copies of DNA that encoded the acetylcholine receptor, complementary messenger RNA molecules in the test tube were synthesized.
- These RNA sequences could be injected into egg cells, which then translated the genetic information into receptor proteins and inserted them into the cell membranes.
- The functional properties of these recombinant receptor channels closely resembled those of end-plate channels at the neuromuscular junction.

Genetic studies

Figure: Genetically engineered frog eggs are useful tools for studying ion channel behavior.

- a) RNA sequences encoding subunits of ion channels can be injected into an egg
- b) The egg will then synthesize the channel proteins and insert them into its surface membrane, where they are accessible for patch clamp experiments



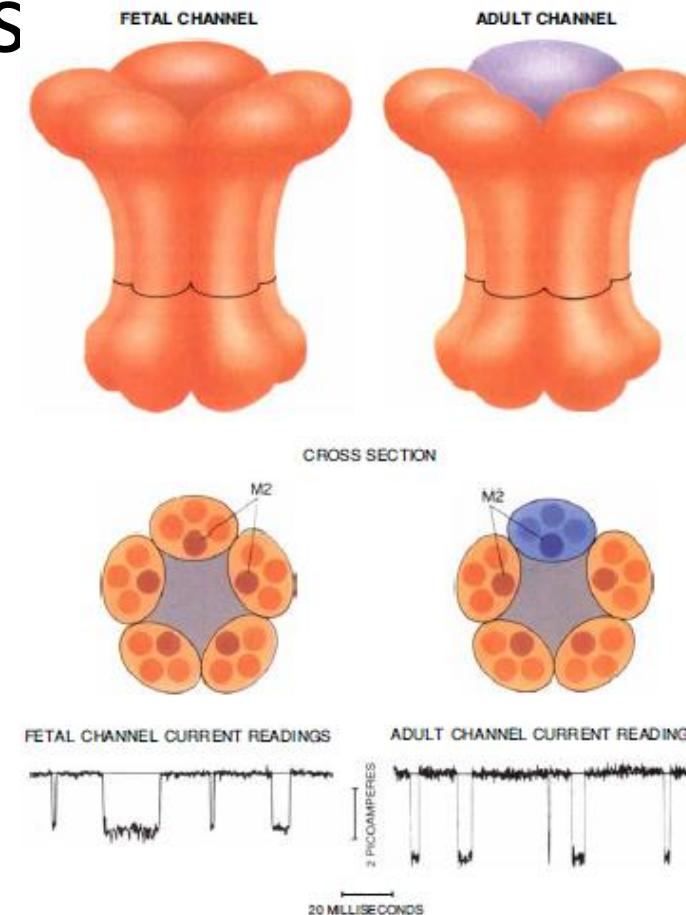
Subtypes of channels

- This combination of techniques helped **to elucidate the structural differences between subtypes of end-plate channels in muscle.**
- **Elementary endplate current recordings** had revealed that mammalian muscle produces **two subtypes of end-plate channels: a low-conductance subtype that appears predominantly in fetal and neonatal muscle and a high-conductance subtype with different gating properties that is expressed in adult muscles.**
- During postnatal development, the fetal subtype gradually disappears and is replaced by the adult subtype
- The **shifting mosaic of channel types** is generated by **changes in the expression of genes that encode the channel subunits.**
- Similar techniques were also used to locate the amino acid sequences in the acetylcholine receptor protein that form the inner wall of the membrane channel.
- An analysis of the **amino acid sequences in the subunits had suggested that they each contained four membrane- spanning segments, designated M1 through M4.**

Structure of Acetylcholine receptor channels

Figure: Structure of acetylcholine receptor channels relates directly to their function.

- Each channel consists of five subunits, each containing four transmembrane segments.
- The M2 segment from each subunit lines the inside of the channel, like a stave in a barrel.
- **Fetal mammals produce an acetylcholine receptor in their neuromuscular end plates that differs in just one subunit from receptors found in adults.**
- Because a fetal channel has a subunit that differs in its M2 segment from that of an adult, the elementary currents that pass through it are smaller.



Genetic studies

- Through recombinant DNA techniques, author created the genes for **chimeric channels**, made up of subunits from species (cows and electric rays) whose own channels had different conducting properties.
- By analyzing the properties of these chimeric channels, it was found that **M2 segment and its neighboring regions contain important determinants of ion transport**.
- Through directed mutations, it was found that three clusters of negatively charged amino acids may form rings at the extracellular and intracellular mouths of the channel
- A similar ring of polar amino acids is present in the transmembrane portion close to the intracellular mouth.
- These negatively charged rings drastically influence the rate of current flow and may select particular ions for transport across the membrane: positively charged sodium and potassium ions would be drawn into the channel, and negatively charged chloride ions would be excluded from it.
- Results also suggested that **the polar amino acids located close to the intracellular part of the M2 transmembrane segment formed the narrowest part of the acetylcholine receptor channel**

Voltage clamp analysis

- Patch pipettes can also be used to study signaling mechanisms at a cellular level, a procedure called voltage clamp analysis.
- Indeed, when measuring events in small cells, the technique has advantages over the use of conventional microelectrodes.
- Conventional voltage clamp analysis has contributed immensely to our understanding of signaling processes in the nervous system and was introduced by Kenneth S. Cole in 1949
- Hodgkin and Huxley used it to unravel the basic mechanisms of nerve excitability.
- The technique involved "taming the axon," as Cole put it, by forcing a transmembrane potential onto the axon of a neuron.
- The resulting membrane currents could then be measured and interpreted.
- Unfortunately, most voltage clamp techniques require that either axial wires or at least two microelectrodes be inserted into a cell, which is generally possible only with the largest types of animal and plant cells.

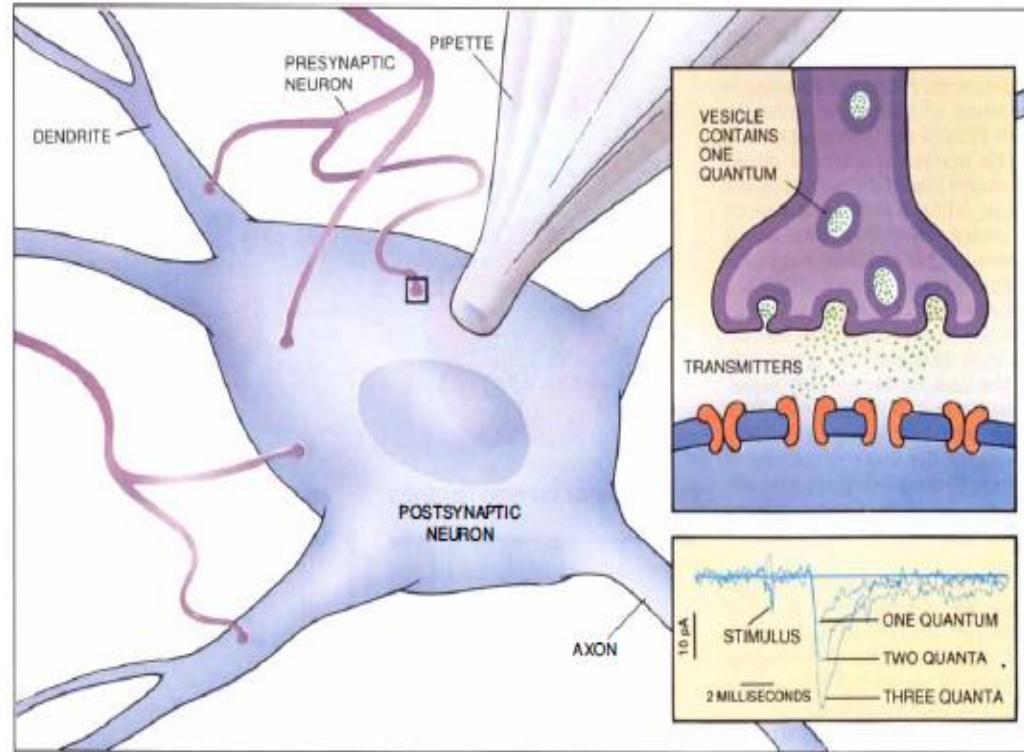
Voltage clamp analysis

- Mammalian cells having diameter of 10 to 30 microns and can barely tolerate impalement with a single standard micro electrode.
- the best understood signals in plant cells were those of giant algae, not those of corn, wheat or sugar beets.
- The ability to create a giga-ohm seal between a patch pipette and a cell membrane literally opened up mammalian and other small cells.
- It made measurements of membrane channels possible, also it enabled researchers to apply voltage clamps to small cells.
- A researcher could rupture the clamped membrane patch without breaking the tight seal between the pipette and the surrounding membrane, thereby obtaining access to the cytoplasm.
- That configuration, termed whole-cell recording, resembles a classic micro electrode impalement.
- It is tolerated by much smaller cells, however, and it provides much better control over the intracellular milieu

Whole-cell recording

Figure:

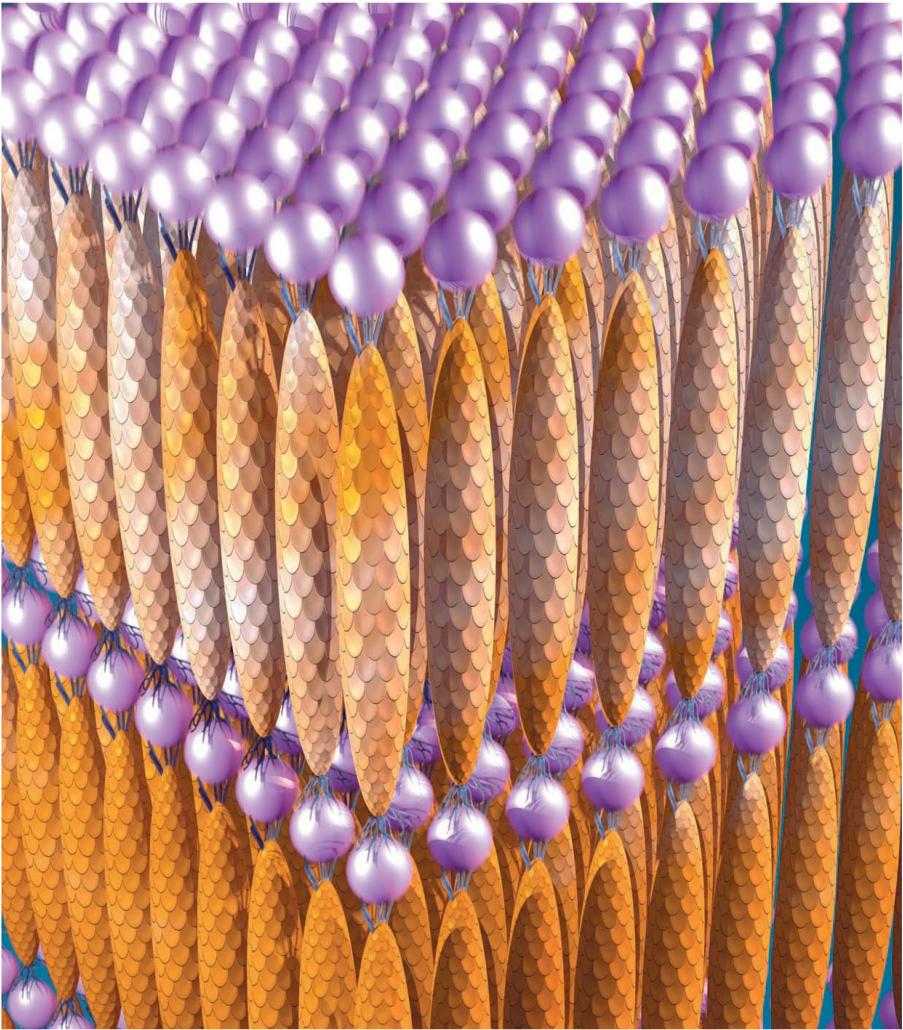
- Whole-cell recording, in which a **patch pipette** takes the place of a conventional microelectrode, has revolutionized the study of mammalian cells, neurons in particular.
- Researchers can work on small slabs of neural tissue containing neurons that are still in contact with their neighbors.
- The technique is sufficiently **sensitive to detect excitatory and inhibitory currents induced in the neurons by individual quanta (packets) of transmitters contained in single presynaptic vesicles** (inset).





Viral Nanoelectronics

M.I. T. breeds viruses that coat themselves in selected substances, then self-assemble into such devices as liquid crystals, nano-wires and electrodes.



3-D LIQUID CRYSTAL depicted in this artist's representation consists of multiple copies of a phage (a bacteria-infecting virus) called **M₁₃** (*gold*) that bound to inorganic nanocrystals (*pink*) and assembled themselves into an ordered array. Such films could be used in flexible displays.

Nano Electronics

- Materials scientists were curious to find how the abalone, a marine snail, constructed its magnificently strong shell from unpromising minerals. (**Angela M. Belcher**)
- In an experiment a thin glass was slipped between the abalone and its shell, then removed.
- A flat pearl was observed.
- Could be used to study shell formation on an hour-by-hour basis, without having to sacrifice the animal.
- It was observed that the abalone manufactures proteins that induce calcium carbonate molecules to adopt two distinct yet seamlessly melded crystalline forms—one strong, the other fast-growing.

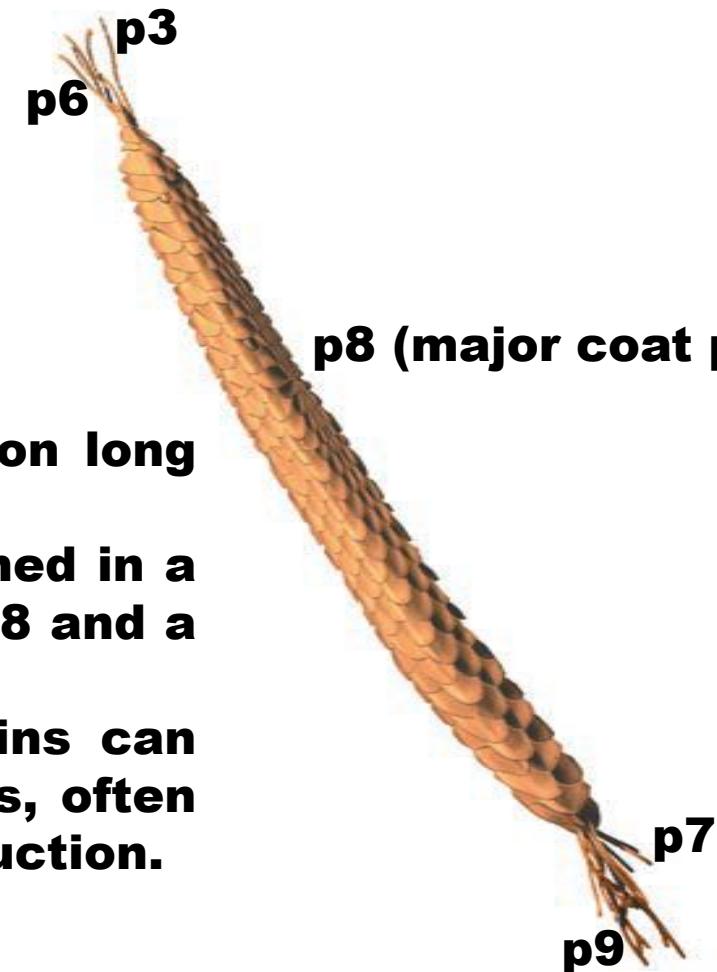
Nano Electronics

- Need for biological agents that could move molecules around like so many bricks, building structures from the ground up, a strategy known in the nanotechnology world as **self-assembly**.
- And, for a critter more tractable than the abalone, is something of a one note small, spry and flexible.
- Earlier monoclonal antibodies were tried because they can be engineered to stick to many different things, but they proved hard to work with.

Nano Electronics(M13 Phage)

- M13 phage, a long, skinny virus that parasitizes bacteria but is harmless to humans.
- Roughly six nanometers wide and a micron long, encloses its single strand of DNA in a protein coat.
- With some 2,700 copies of one kind of protein lining the filamentous body.
- And a few copies each of several other kinds of protein capping the ends.

M13 VIRUS



- **Long and Skinny.**
- **The M13 virus is about a micron long and six nano-meters wide.**
- **Its single loop of DNA is sheathed in a coat composed of the protein p8 and a smattering of other proteins.**
- **Variations in any of the proteins can alter the properties of the virus, often in ways useful to device construction.**

Nano Electronics(M13 Phage)

- The different kinds of proteins can be engineered to vary from phage to phage, making for a billion possible permutations, each conferring particular chemical affinities.
- A phage might stick to one material along its sides, another at one end, and still another at the opposite end.
- Chemical specificity has long been exploited by biologists, who use M13 phages that bind to particular organic substances to identify unknown samples.

Nano Electronics(M13 Phage Features)

- It was shown that the virus could also tag and manipulate inorganic molecules, such as the metals and semiconductors that lie at the heart of so many useful products.
- A process called **directed evolution** was used to get a phage that binds to the right molecule.
- Amplification could provide with trillions of copies of a promising subset of phages for another stage of evolution.
- Thus, the conditions in the solution altered to make it a little harder for the phages to bond to the target material.

Nano Electronics(M13 Phage Features)

- The less sticky variants are washed off, the survivors are amplified, and the process is repeated under still more demanding conditions.
- At the end of the process, only one phage variant remained—the most selectively sticky of them all.
- **Example-** A phage was with a highly specific taste for gold was put into a solution containing gold ions.
- It gilded itself into a wire—let a micron long, suitable for connecting adjacent elements in a microcircuit.

Nano Electronics(M13 Phage Features)

- A variant of this phage **can** even link up with its fellows to form a gold wire many centimeters long, which can be spun like thread and woven into cloth fabric.
- Such a wire, bonded to chemically sensitive receptors, might detect toxic or biologically threatening agents.
- The experimental merit of various other organisms have been examined so far.
- M13-- because its immense length-to width ratio makes it naturally assemble into more complex shapes.

Nano Electronics(M13 Phage Features)

- M13 phages were selected to form a film 10 centimeters square and less than a micron thick,
- The structure was then fixed into a stable sheet by throwing in the odd chemical cross-linkage.
- **Applications**-- developing such sheets as electrodes for an ultralight lithium-ion battery.
- The negative electrode can be formed from a sheet of phages bred to encrust themselves in gold and cobalt oxide.
- The gold was used to increase conductivity, the cobalt oxide to exchange ions with the battery electrolyte.

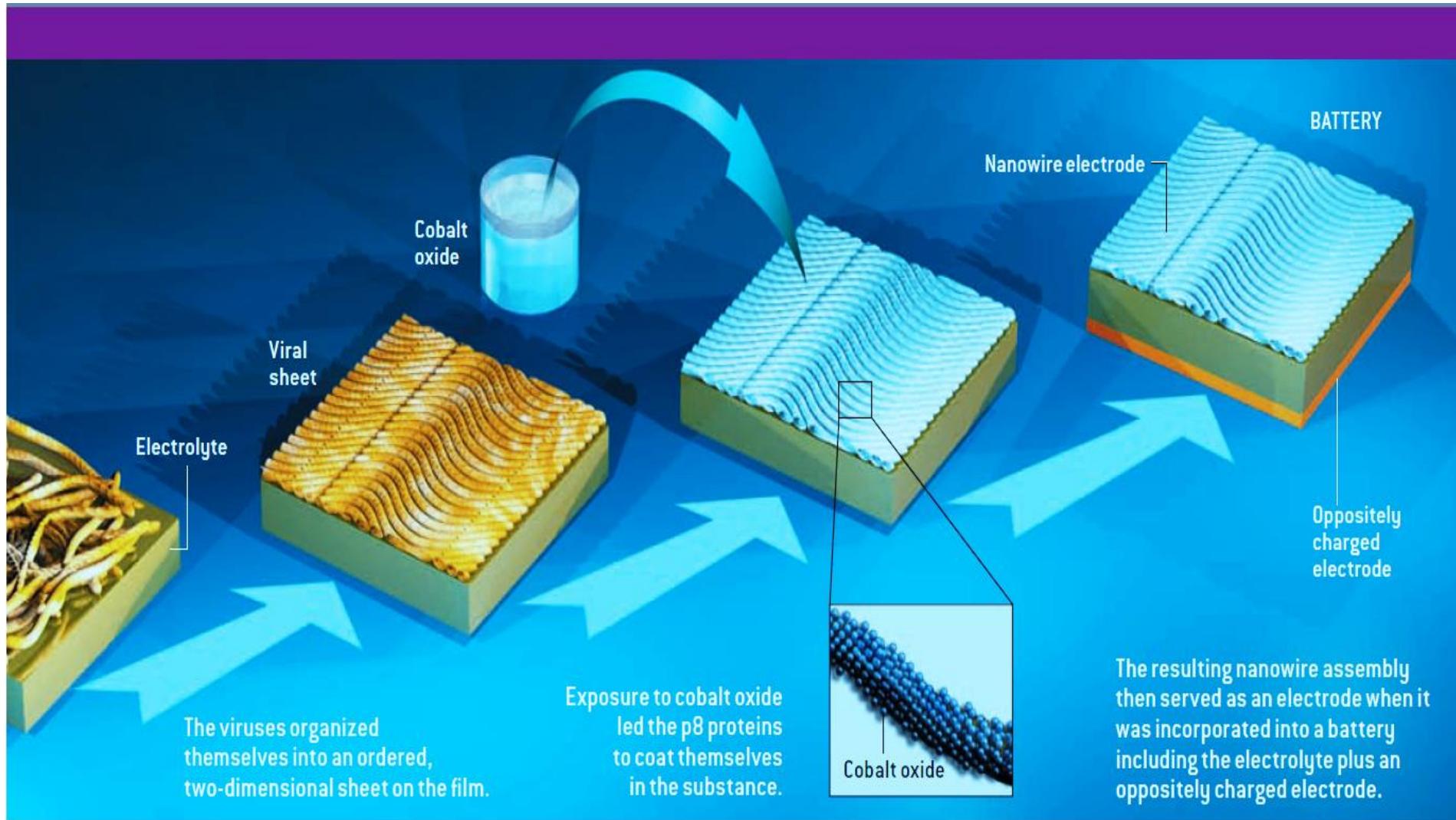
A NEW KIND OF BATTERY



- The experiment depicts **M₁₃ variants as building blocks for electrodes in flexible lightweight lithium-ion batteries.**
- An **M₁₃ variant was selected that can bind to cobalt oxide, a substance able to store lithium ions.**
- Copies of the virus was put on an electrolyte film containing lithium.

Nano Electronics(M13 Phage Features)

- Such ion exchange is what moves charge from one electrode to the other.
- The electrode assembles directly on a prepatterned polymer electrolyte, forming a bilayer.
- Phages were also grown to obtain a positive counter electrode that will stick to the other side of the electrolyte.
- The goal was to shape the sheets into a solid with positive and negative electrodes alternating on the surface so they may be connected in series for higher voltage.



Nano Electronics(M13 Phage Features)

- The short distances between electrodes permit fast charging and discharging and allow for the optimal use of components.
- **Specificity-** One phage is specific for the semiconductor gallium arsenide and is insensitive to its close cousin gallium nitride, giving it a power of discrimination that might allow it to detect flaws in chips.
- Chipmakers sometimes grow crystals of one of these substances on top of some other semiconductor so that the slightly different spacing of the crystalline lattices will induce mechanical strain, which in turn will affect electronic behaviour.

Nano Electronics(M13 Phage Features)

- When the crystals do not mesh properly, the occasional atom will jut out where it should not, creating a defect to which a phage can stick.
- If such a phage also bears a fluorescent tag, it will then glow under the right conditions, and a microscope can pinpoint the defect.

Nano Electronics (Future Research)

- Coax M13 phages into building complete transistors from molecules of semiconductors.
- Getting M13 to bind both to cancer cells and to nanodevices known as quantum dots that show up in medical body scans.
- Flexible screens.

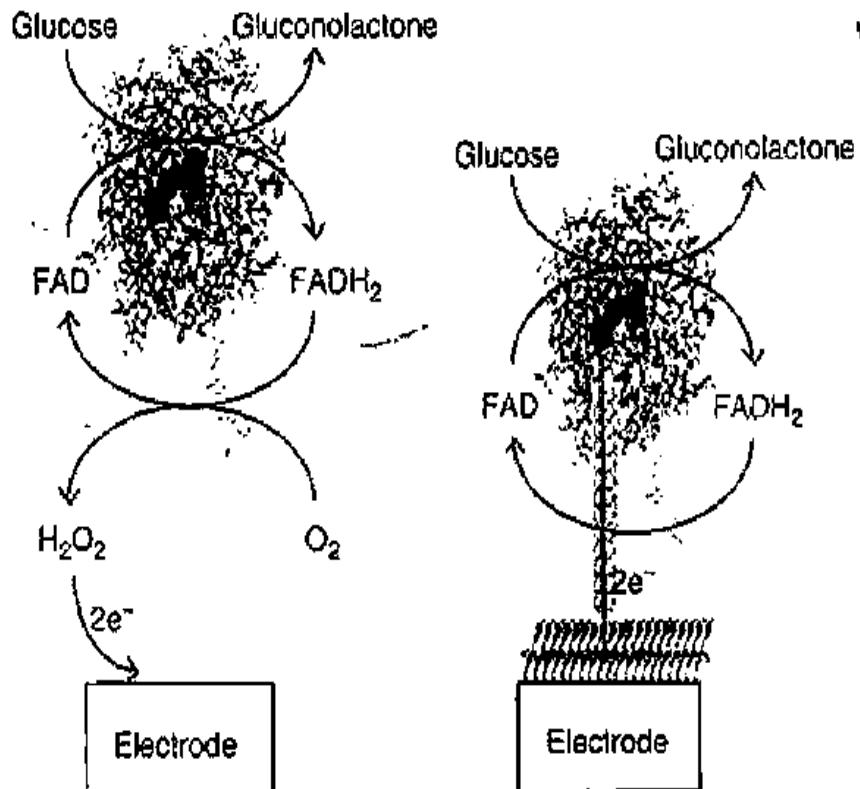
Carbon Nanotubes

Towards the Next -Generation of
Enzyme Biosensors

1. Introduction

- **Enzymatic Biosensors-** One of the opportunities that nanomaterials provides is a more efficient way of communicating the activity of biological molecules used in biosensors.
 - This communication is achieved via the transfer of electrons.
 - In the case of many devices, electrons must be transferred between redox protein and an electrode over which the proteins are immobilized.
 - This communication between redox proteins and electrodes can be further improved using carbon nanotubes.
 - Efficient transfer of electrons will result in improved biosensing and bioelectronic devices.
 - Example: Enzyme GOx an oxido-reductase enzyme that oxidizes glucose to gluconolactone.

1. Introduction

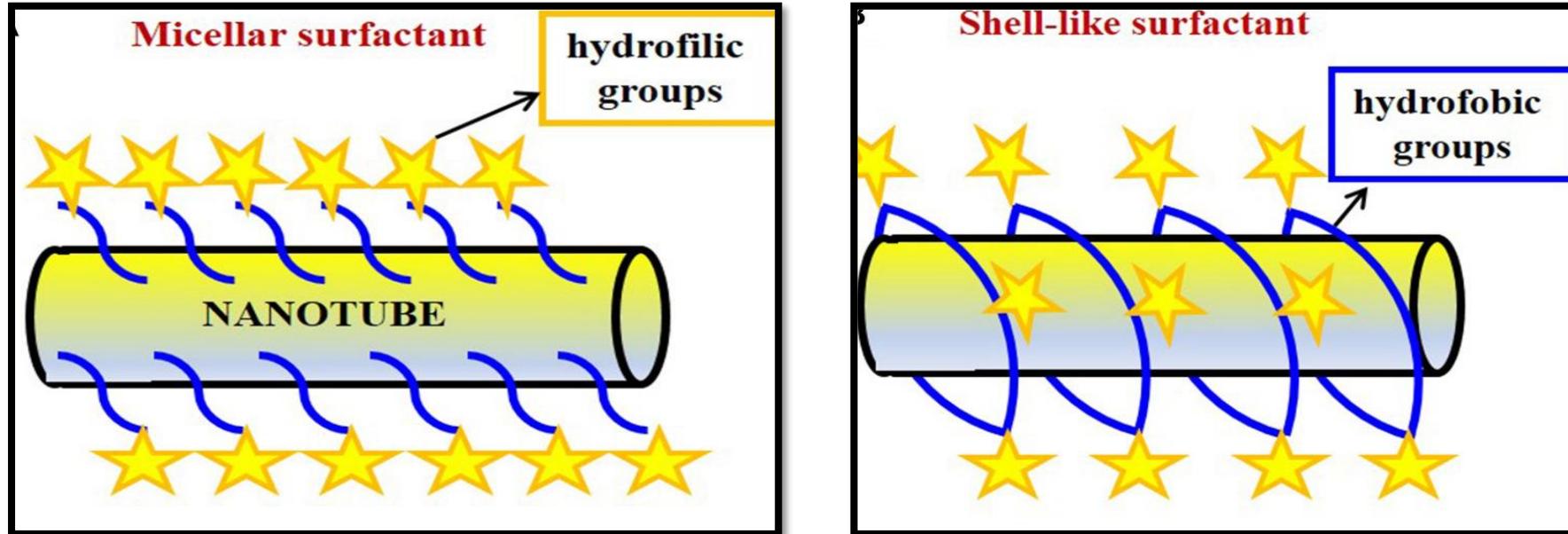


- A classical first generation enzyme electrode using GOx as an example where Glucose is oxidized to gluconolactone and in the process the redox-active center of GOx, FAD to reduced to FADH₂.
- The FADH₂ is then oxidized back to FAD by freely diffusing oxygen.
- The oxygen is reduced to hydrogen peroxide which is detected at the electrode.
- A carbon nanotube is plugged into the enzyme and the reoxidation of the FADH₂ is achieved via direct electron transfer.

1. Introduction

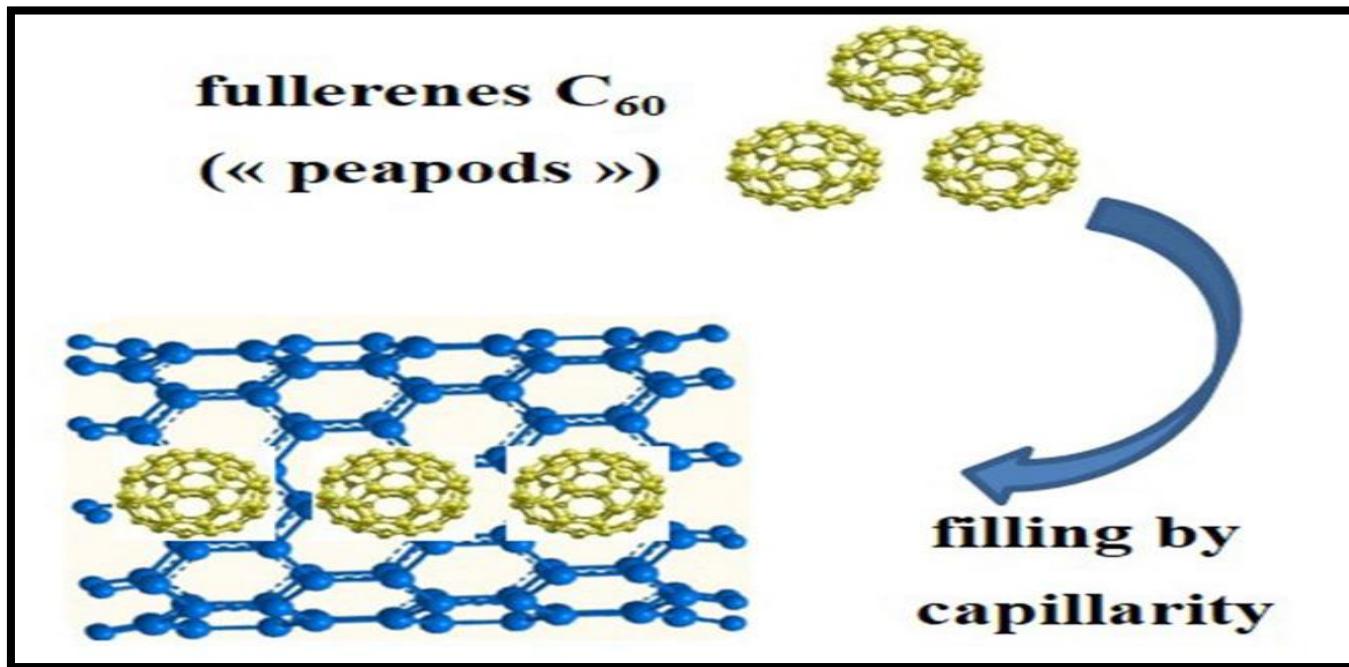
- In many commercial glucose biosensors the O_2/H_2O_2 couple is replaced by a synthetic mediator such as ferrocene.
- But the principle of a diffusing species shuttling electrons between the protein and an electrode is the same .
- The advantage of CNTs compared to other nanomaterials lies in unique combination of electrical, magnetic, optical, mechanical, and chemical properties, which offer great promises for a wide range of applications, including biosensing.
- CNTs can serve as platforms to conjugate other compounds at their surface (exohedral functionalization).
- CNTs shells can be opened and filled (endohedral functionalization) without losing their stability.
- Functionalized CNTs can effectively cross biological barriers such as the cell membrane and penetrate individual cells.

2. Exohedral and endohedral functionalization of CNTs.



Non-covalent surface functionalization of a bucky tubes with a micellar surfactant or with a shell-like surfactant wrapping around the nanotubes

2. Exohedral and endohedral functionalization of CNTs.

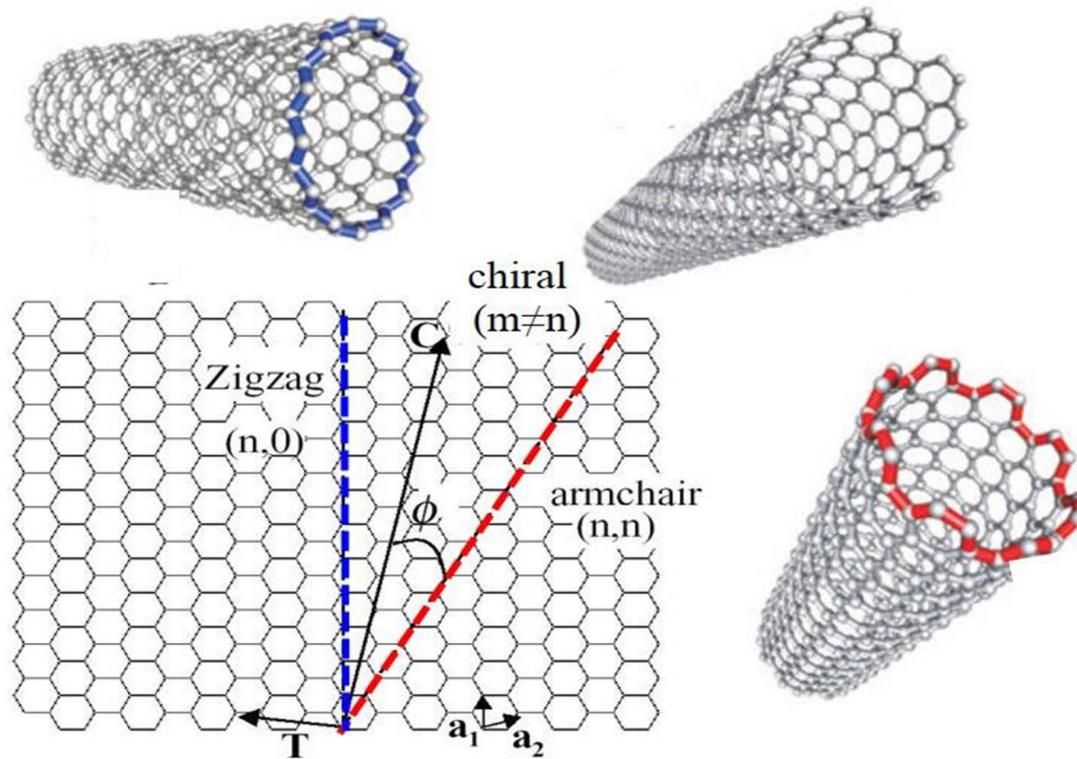


Fullerenes can be used for capping the ends or filling the capillaries

3. Basics of Carbon Nanotubes and Their Electrochemistry

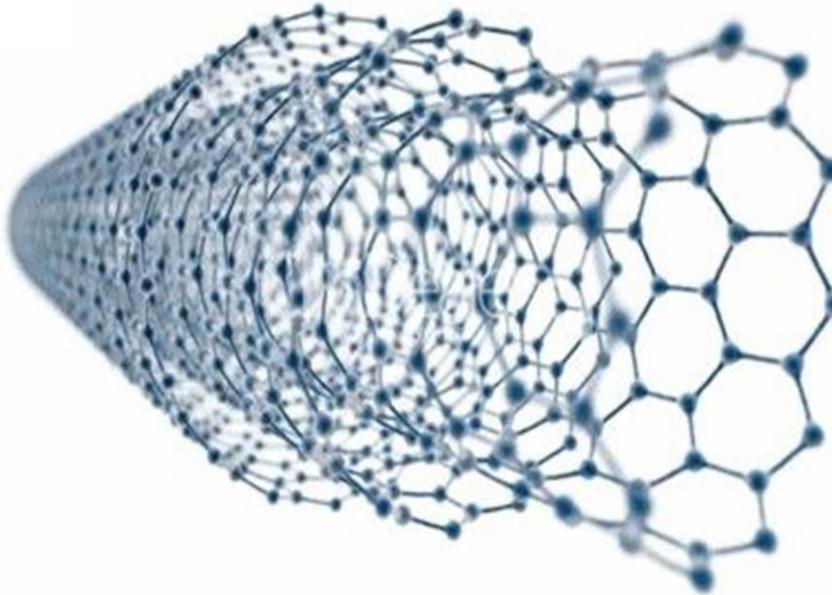
- The nanotubes are electrocatalytic allowing electrochemistry of certain biologically important species to be detected at much lower potential than other electrodes.
- The nanotubes can have lengths ranging from tens of nanometers to several microns.
- Depending on their number of walls, CNTs are designated single walled (SWNTs) or multi-walled (MWNTs).
- SWNTs, as the name suggests, consist of a single hollow tube with diameters between 0.4 to 2 nm,
- whereas MWNTs are composed of multiple concentric nanotube 0.34 nm apart, where the final MWNT has diameters of 2-100 nm.
- Carbon nanotubes can be thought of as all sp^2 - carbons arranged in graphene sheets that have been rolled up to form a seamless hollow tube.
- The tubes can be capped at the ends by a fullerene-type hemisphere, but are often open.

3. Basics of Carbon Nanotubes and Their Electrochemistry



Single-walled Carbon nanotubes (SWNTs) structures in function of their chirality (zigzag, armchair, and chiral).

3. Basics of Carbon Nanotubes and Their Electrochemistry



Structure of multi-walled carbon nanotubes (MWNTs) made up of several concentric shells.

3. Basics of Carbon Nanotubes and Their Electrochemistry

- The small size of SWNT important for interfacing with proteins if the nanotubes are to penetrate the outer shell of the protein with a minimization of any disruption of the protein structure.
- For example, the entrance on the surface of GOx to the active site is approximately 1 nm in diameter and therefore MWNT with are significantly greater in size than this are unlikely to be effectively integrated with the protein.
- SWNTs can be metals, semiconductors. or small-band-gap semiconductors depending on their diameter and chirality.
- The chirality of the SWNT relates to the angle at which the graphene sheets roll up and hence the alignment of the π -orbitals.
- In SWNTs with a small diameter, approximately two-third exhibit semiconducting properties and one-third are metallic.

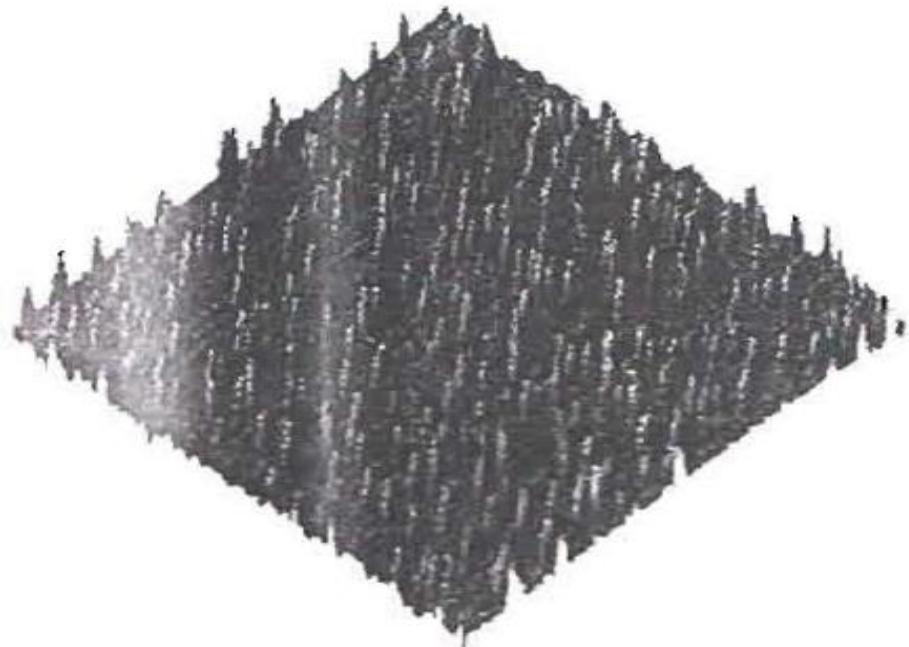
3. Basics of Carbon Nanotubes and Their Electrochemistry

- Carbon nanotubes are anisotropic, having two physically and chemically distinct regions: the walls and the ends.
- Electrochemically active regions are at the open ends of the CNTS which posses variety of oxygenated species.
- There are two main approaches to forming aligned carbon nanotube arrays.
- The first is to grow aligned nanotubes directly from a surface- typically generates MWNT that are too large to plug to proteins.
- New approaches have been developed to grow SWNT directly from a surface.
- The alternative approach is to align the carbon nanotubes by self-assembly. (mostly preferred)
- The SWNTs were shortened in mixture of concentrated sulfuric acid and concentrated nitric acid in 3:1 ratio.

3. Basics of Carbon Nanotubes and Their Electrochemistry

- This oxidative shortening leaves several carboxylic groups at either ends of the tubes.
- The carboxylate ends can be converted to carbodiimide leaving groups using 1 g dicyclohexylcarbodiimide (DCC).
- This allows reaction with amines to form an amide bond.
- Exposure of shortened nanotubes which have had the carboxylic acid groups activated with DCC to a gold electrode modified with a self assembled monolayer of cysteamine results in one end of the nanotubes attaching to the gold electrodes.
- This gives an array of SWNTs standing normal to the surface and can be viewed using Atomic force microscopy (AFM).

3. Basics of Carbon Nanotubes and Their Electrochemistry



**A tapping mode AFM
image of aligned carbon
nanotube array, formed
by self assembly.**

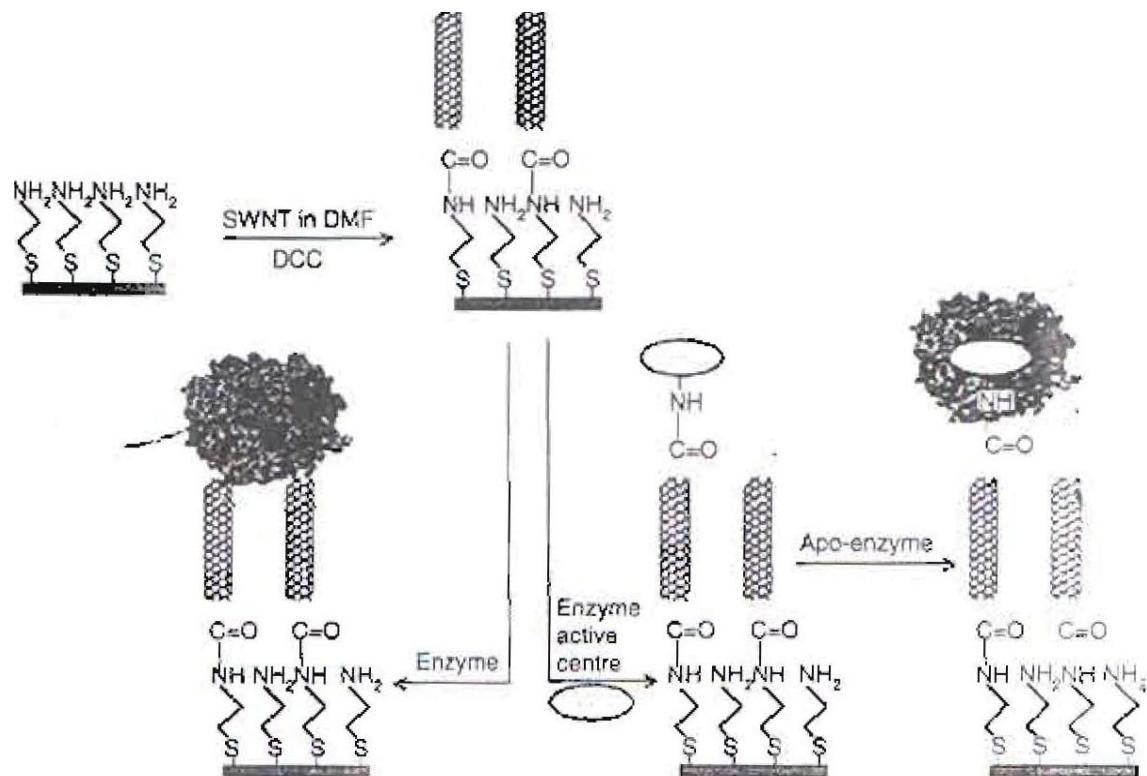
Fabrication of enzyme electrodes

- Nano scale extension of bulk electrode sufficient small to penetrate into proteins (enzymes).
- The distance that electrons must tunnel from redox active center to the electrode is shortened by electrical connections.
- Enzymes having redox active centers close to the surface of proteins (enzymes) e.g. MP 11 made of only 11 amino acids and heme redox active center.
- It is obtained by proteolytic cleavage of horse heart cytochrome c.

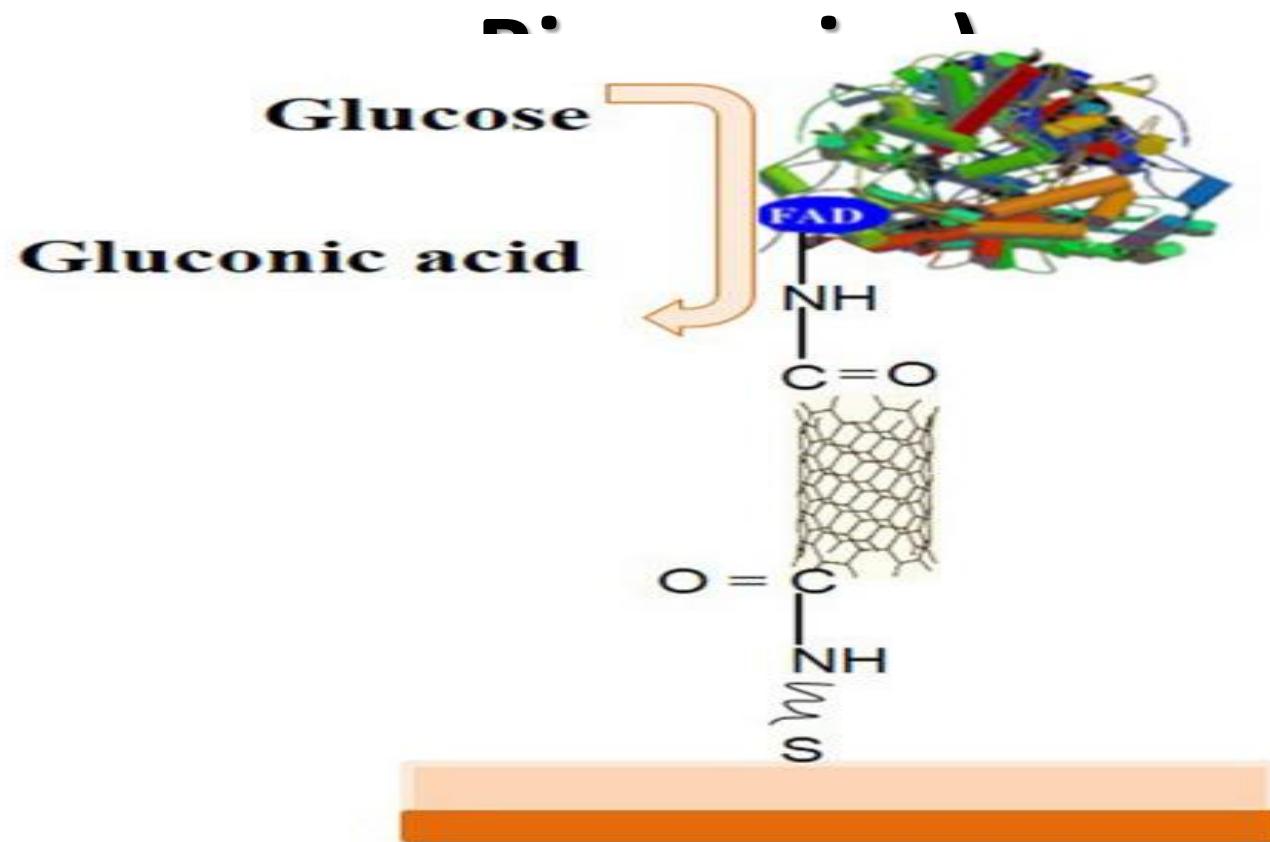
Integrating CNTs with Proteins where redox center is embedded deep within the proteins (Glucose oxidase)

- Chance of penetration of nanotube into protein redox center.
- Distance between FAD and Surface of enzyme (13 Å).
- Enzyme is reconstituted over the end of nanotubes (SWNTs)

Schematics of the fabrication protocol used to form enzyme electrode covalently attached to ends of SWNT electrode array

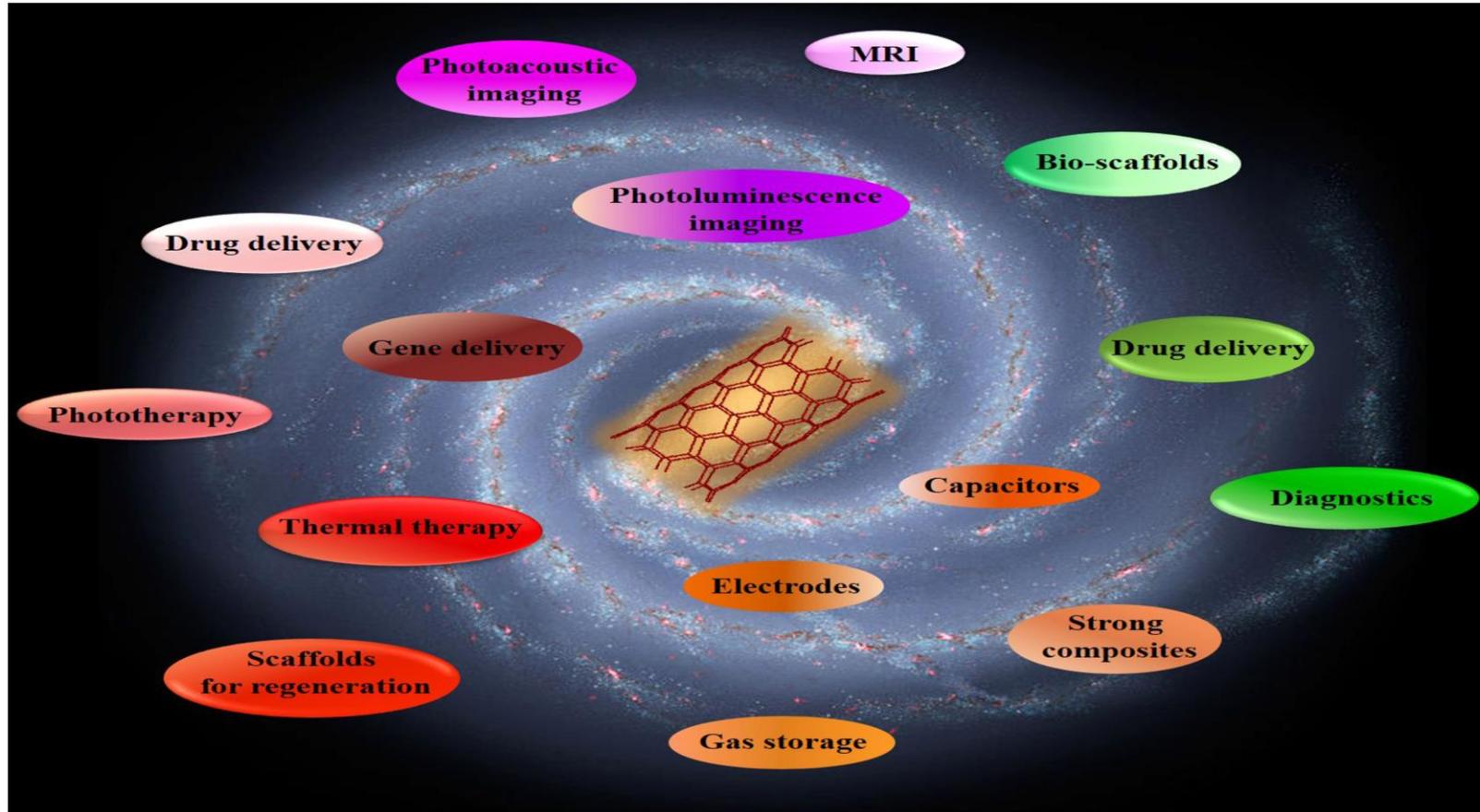


4. APPLICATIONS OF CNT (Examples-

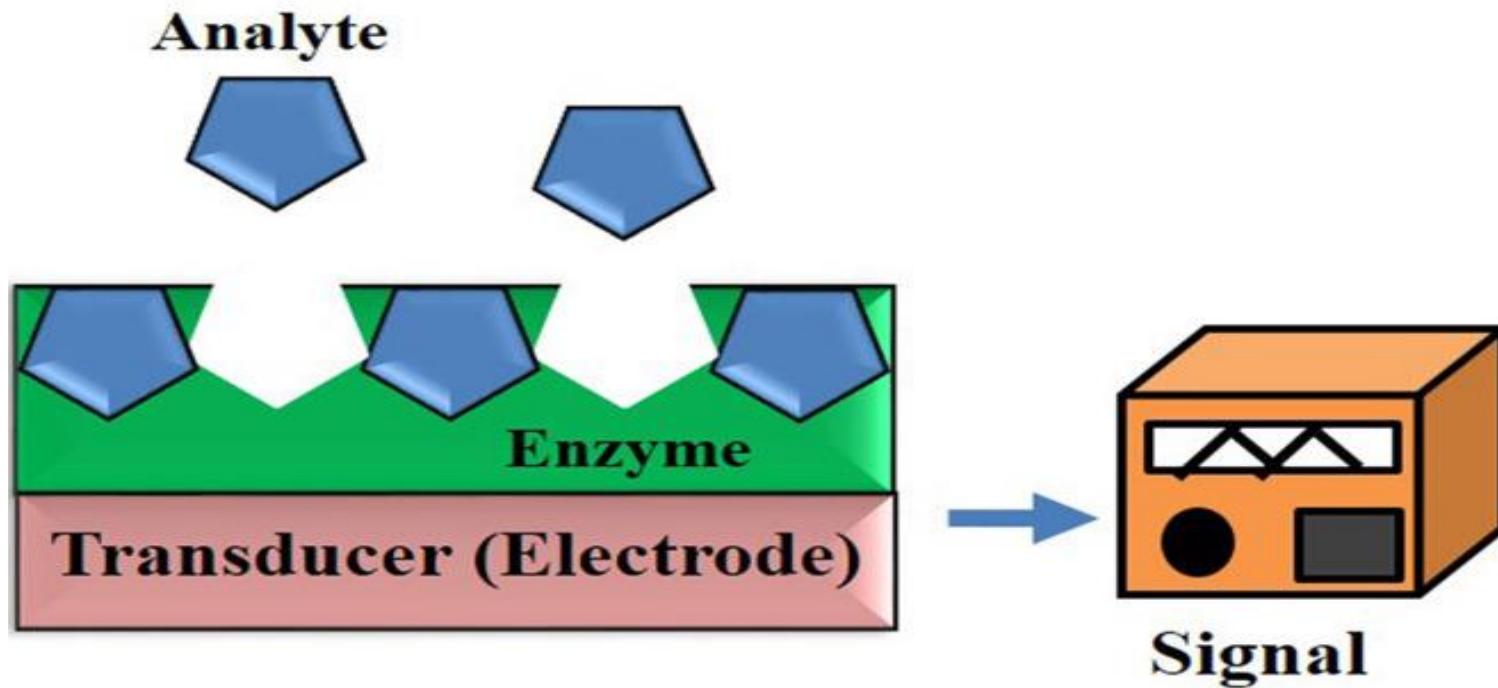


SWNT electrically-contacted glucose oxidase electrode.

4. Applications of CNT

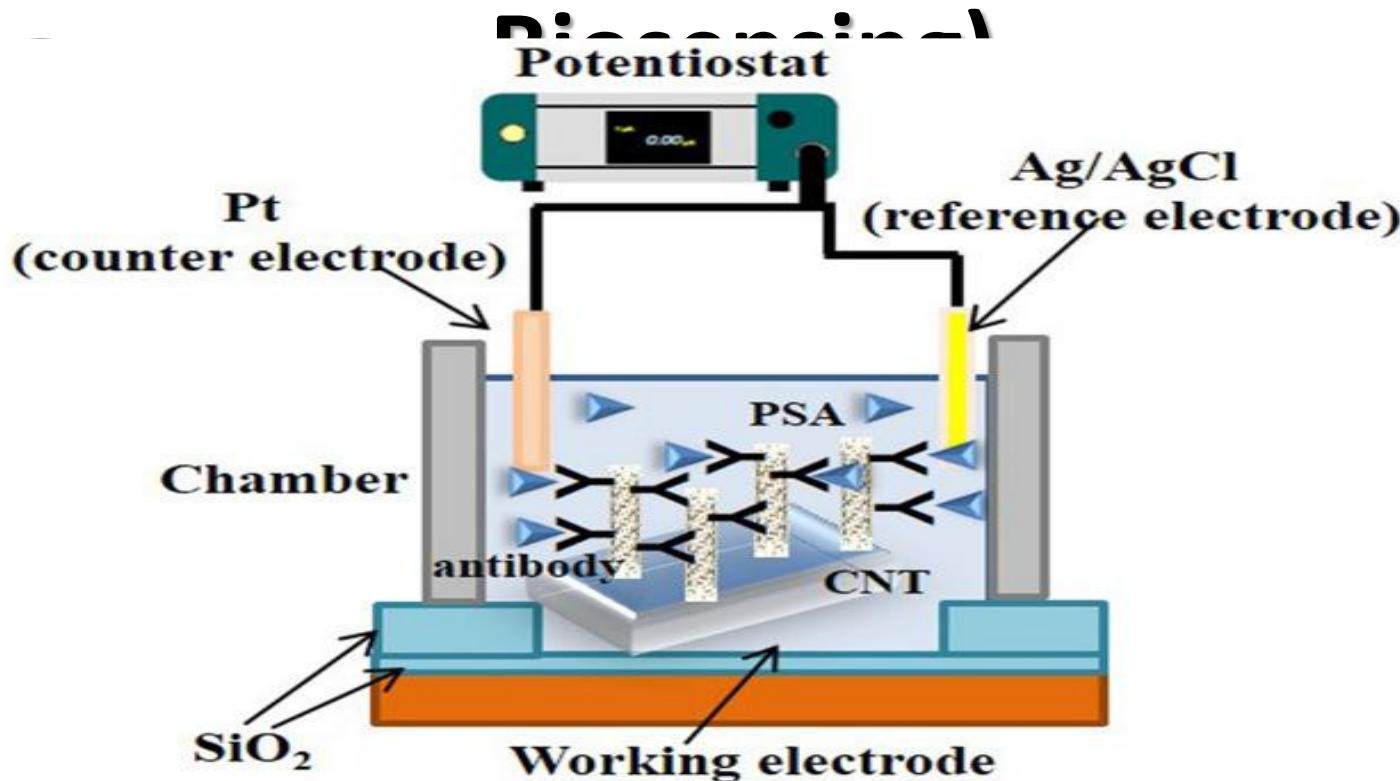


4. APPLICATIONS OF CNT (Examples- Biosensing)



Typical design of an enzyme-based electrochemical biosensor.

4. APPLICATIONS OF CNT (Examples-



Schematic illustration of a label-free amperometric biosensor for PSA detection.

Luminescent Quantum Dots for Biological Labeling

BBL 747
Bionanotechnology

Overview

- The integration of nanotechnology with biology and medicine is expected to produce major advances in medical diagnostics, therapeutics, molecular biology, and bioengineering
- Recent advances have led to the development of functional nanoparticles (electronic, optical, magnetic, or structural) that are covalently linked to biological molecules such as peptides, proteins, and nucleic acids
- Due to their size-dependent properties and dimensional similarities to biomacromolecules, these bioconjugates are well suited as contrast agents for *in-vivo* magnetic resonance imaging (MRI), as nanoscale carriers for drug delivery, and as nanostructured coatings and scaffolds for medical implants and tissue engineering
- In comparison with organic dyes and fluorescent proteins, **semi-conductor quantum dots (QDs) represent a new class of fluorescent labels with unique advantages and applications.**
- For example, the **fluorescence emission spectra of QDs can be continuously tuned by changing the particle size, and a single wavelength can be used for simultaneous excitation of all different-sized QDs**

Quantum Dots

- Quantum dots (QDs), often described as ‘artificial atoms,’ exhibit discrete energy levels, and their bandgap (distance between the valance band of electrons and conduction band) can be precisely modulated by varying the size .
- QDs are nanometer-scale semiconductor crystals composed of groups II to VI or III to V elements and are defined as particles with physical dimensions smaller than the exciton Bohr radius
- QDs exhibit unique luminescence characteristics and electronic properties such as wide and continuous absorption spectra, narrow emission spectra, and high light stability .
- They absorb white light and then re-emit a specific color a few nanoseconds later depending on the bandgap of the material .



Figure: Ten distinguishable emission colors of ZnS-capped CdSe quantum dots excited with a near-UV lamp. From left to right (blue to red), the emission maxima are located at 443, 473, 481, 500, 518, 543, 565, 587, 610, and 655 nm.

Preparation of QDs

- Surface-passivated QDs are highly stable against photobleaching and have narrow, symmetric emission peaks (25–30 nm full width at half maximum).
- It has been estimated that CdSe quantum dots are about 20 times brighter and 100 times more stable than single rhodamine 6G molecules
- Semiconductor QDs (e. g., CdSe, CdTe, CdS, ZnSe, InP, and InAs) are most often composed of atoms from groups I–VII, II–VI, or III–V elements.
- Earlier attempts to synthesize QDs were conducted in aqueous environments with stabilizing agents such as thioglycerol and polyphosphate.
- However, the resulting QDs showed poor quantum yields (<10 %) and broad size distributions (relative standard deviation RSD > 15 %). Quantum yield is a ratio of the number of photons emitted to the number of photons absorbed.
- A high-temperature organometallic procedure for QD synthesis yields near-perfect nanocrystals with quantum yields as high as 50% at room temperature, and a particle size distribution as narrow as 5 %.

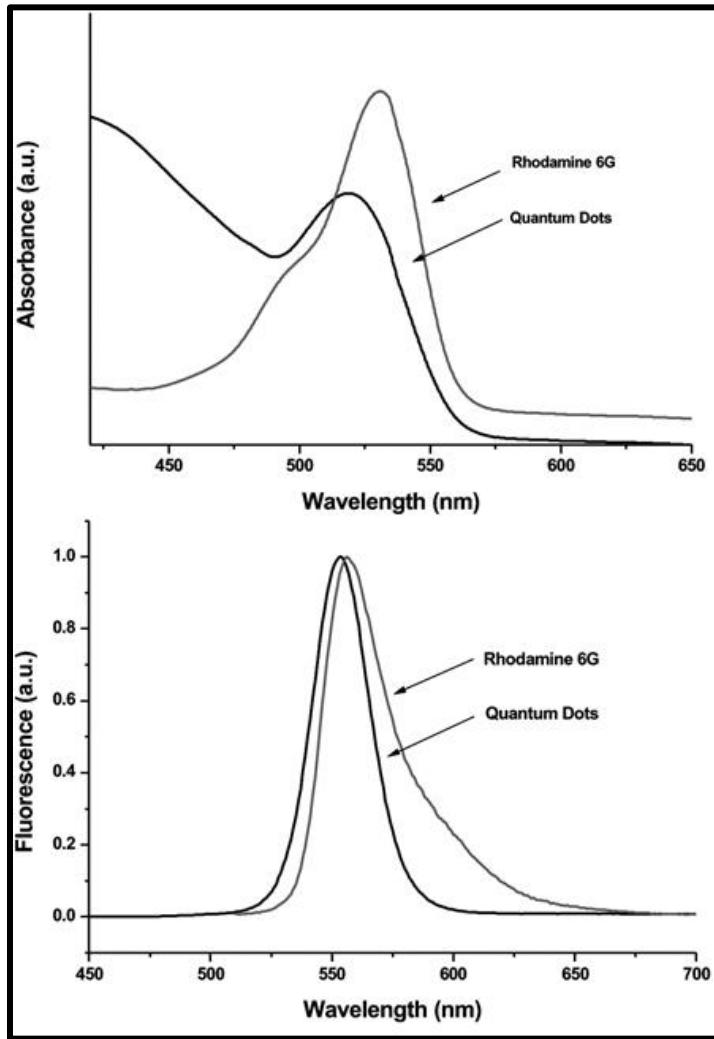
Preparation of QDs

- To prepare **type II–VI QDs**, a metal precursor (such as dimethyl cadmium) and a **chalcogenide compound** (such as selenium) were first dissolved in **tri-n-butyl phosphine (TBP)** or **tri-n-octyl phosphine (TOP)**, and are then **injected into a hot coordinating solvent such as tri-n-octylphosphine oxide (TOPO)** at 340–360 °C.
- Studies conducted, have shown that high-quality nanocrystals could also be prepared by using CdO as an inexpensive starting material
- The **nanocrystal size can be tuned by heating QDs in TOPO at 300 °C for an extended period of time** (ranging from seconds to days, depending on the desired particle size), in which the QDs grow by **Ostwald ripening**.
- In this process, **smaller nanocrystals are broken down**, and the **dissolved atoms are transferred to larger nanocrystals**.
- **The rate of growth is dependent upon temperature** and the amount of limiting reagents.
- Alternately, **continuous injection of organometal/chalcogenide precursors at 300 °C can be used to increase the size of QDs**

Passivation of QDs

- For improved optical properties, the QDs are often coated and passivated by a thin layer of a higher bandgap material. For example, the fluorescence quantum yields of CdSe QDs increase from 5% to 50% with one to two monolayers of ZnS capping
- At present, ZnS and CdS are most commonly used to cap CdSe QDs. The bandgap energy of bulk CdS is about 0.9 eV higher than that of CdSe, while the ZnS and CdSe bond lengths are similar; these conditions lead to the epitaxial growth of a smooth ZnS layer on the surface of CdSe core particles. Similar procedures have been used to synthesize group III–V nanocrystals such as InP and InAs
- Epitaxy refers to the deposition of an overlayer on a crystalline substrate.
- Semiconductor QDs absorb photons when the energy of excitation exceeds the bandgap energy. During this process, electrons are promoted from the valence band to the conduction band.
- Measurements of UV-Visible spectra reveal a large number of energy states in QDs. The lowest excited energy state is shown by the first observable peak (also known as the quantum-confinement peak), at a shorter wavelength than the fluorescence emission peak.

Figure: Comparison of the excitation (top) and emission (bottom) profiles between rhodamine 6G and CdSe quantum dots.



Overview

- Excitation at shorter wavelengths is possible because **multiple electronic states are present at higher energy levels**. In fact, the molar extinction coefficient gradually increases toward shorter wavelengths. This is an important feature for biological applications because **it allows simultaneous excitation of multicolor QDs with a single light source**.
- **Light emission arises from the recombination of mobile or trapped charge carriers.** The emission from mobile carriers is called “**excitonic fluorescence**”, and is observed as a sharp peak.
- The emission spectra of single ZnS-capped CdSe QDs are as narrow as 13 nm (full width at half maximum or FWHM) at room temperature
- **Defect states in the crystal interior or on its surface can trap the mobile charge carriers** (electrons or holes), leading to a **broad emission peak that is red-shifted from the excitonic peak**.
- **Nanocrystals with a large number of trap states generally have low quantum yields**, but surface **capping or passivation can remove these defect sites and improve the fluorescence quantum yields**.
- The excitonic fluorescence is dependent on the nanocrystal size. It has been demonstrated that an **approximately linear relationship between the particle size and the bandgap energy**

Overview

- This **quantum-size effect is similar to that observed for a “particle in a box.”** Outside of the box, the potential energy is considered to be infinitely high. Thus, mobile carriers (similar to the particle) are confined within the dimensions of the nanocrystal (similar to the box) with discrete wavefunctions and energy levels. As the physical dimensions of the box become smaller, the bandgap energy becomes higher.
- For CdSe nanocrystals, the sizes of 2.5 nm and 5.5 nm correspond to fluorescence emission at 500 nm and 620 nm, respectively.
- In addition to size, the **emission wavelength can be varied by changing the semiconductor material.** For example, InP and InAs QDs usually emit in the far-red and near-infrared, while CdS and ZnSe dots often emit in the blue or near-UV
- Also, **elongated QDs (called quantum rods) show linearly polarized emission,** whereas the fluorescence emission from spherical CdSe dots is either circularly polarized or not polarized
- In comparison to organic dyes such as rhodamine 6G and fluorescein, CdSe nanocrystals show similar or slightly lower quantum yields at room temperature. The lower quantum yields of nanocrystals are compensated by their **larger absorption cross-sections and much reduced photobleaching rates.**

Overview

- It was estimated that **molar extinction coefficients of CdSe QDs are about 10^5 to $10^6 \text{ M}^{-1} \text{ cm}^{-1}$** , depending on the particle size and the excitation wavelength. These values are **10- to 100-fold larger than those of organic dyes**, but are similar to the absorption cross-sections of phycoerytherin, a multi-chromophore fluorescent protein.
- It has been estimated that **single ZnS capped CdSe QDs are ~ 20 times brighter than single rhodamine 6G molecules**. Similarly, phycoerytherin is estimated to be 20 times brighter than fluorescein
- Another attractive feature of using QDs as biological labels is their high photostability. The photobleaching rate of silica-coated ZnS-capped CdSe QDs was examined against that of rhodamine 6G.
- The **QD emission stayed constant for 4 hours, while rhodamine 6G was photobleached after only 10 minutes**.
- It has been suggested that capped **CdSe nanocrystals are 100- to 200-fold more stable than organic dyes and fluorescent proteins**. Under intense UV excitation, single phycoerytherin molecules are found to photobleach after 70 seconds, while the fluorescence emission of quantum dots remain unchanged after 600 seconds
- The **photobleaching of QDs arise from a slow process of photo-induced chemical decomposition**. Henglein and coworkers speculated that CdS decomposition is initiated by the formation of S or SH radicals upon optical excitation. These radicals can react with O₂ from the air to form a SO₂ complex, resulting in slow particle degradation.

Overview

- Single QDs have been shown to **emit photons in an intermittent on-off fashion**, similar to a “**blinking**” behavior reported for single fluorescent dye molecules, proteins, polymers, and metal nanoparticles.
- The fluorescence of single **QDs turns on and off at a rate that is dependent on the excitation power**. This phenomenon has been suggested to arise from a light-induced process involving **photoionization and slow charge neutralization** of the nanocrystals
- When two or more electron-hole pairs are generated in a single nanocrystal, the energy released from the combination of one pair could be transferred to the remaining carriers, one of which is preferentially ejected into the surrounding matrix.
- Subsequent photogenerated electron-hole pairs transfer their energy to the resident, unpaired carrier, leading to nonradiative decay and dark periods.
- The luminescence is restored only when the ejected carrier returns to neutralize the particle.
- Thermal trapping of electrons and holes is also believed to be a contributing factor because **a dependence of the blinking rate on temperature** was observed.
- A further finding is that single dots exhibit random fluctuations in the emission wavelength (spectral wandering) over time. This effect is attributed to interactions between excitons with optically induced surface changes

Biological Applications of QDs

- In order to exploit the novel optical properties of QDs for biological applications, a number of methods have been reported for converting hydrophobic QDs to water-soluble and biocompatible nanocrystals
- In one approach, mercaptopropyl trimethoxysilane (MPTMS) adsorbs onto the QD surface, and displaces the surface-bound **tri-n-octylphosphine oxide** (TOPO) molecules.
- A silica-shell is formed on the surface by introduction of a base and then hydrolysis of the MPTMS silanol groups. The polymerized **silanol groups help stabilize nanocrystals against flocculation, and render the QDs soluble in intermediate polar solvents** such as methanol and dimethylsulfoxide.
- Further reaction of **bifunctional methoxy molecules, such as aminopropyltrimethoxysilane and trimethoxysilyl propyl urea, makes the QDs more polar and soluble in aqueous solution.**
- In another method, bifunctional molecules such as **mercaptoacetic acid and dithiothreitol are directly adsorbed onto the QD surface**. Mercapto compounds and organic bases are added to TOPO-QDs dissolved in organic solvents. The **base deprotonates the mercapto functional group** and carboxylic acid (in the case of mercaptoacetic acid), which leads to a favorable **electrostatic binding between negatively charged thiols and the positively charged metal atoms.**

Methods

- The QDs precipitate out of solution and can be redissolved in aqueous solution (pH 5). The presence of highly polar functional groups, such as –COOH, –OH, or –SO₃Na (from bifunctional mercapto molecules) makes the nanocrystals soluble in water.
- A third approach **for linking biomolecules onto the particle's surface is to use an exchange reaction,**
- **in which mercapto-coated QDs are mixed with thiolated biomolecules (such as oligonucleotides and proteins).**
- After overnight incubation at room temperature, a chemical equilibrium is reached between the thiolated molecules in solution and on the QD surface. This method has been used to adsorb oligonucleotides and biotinylated proteins onto the surface of QDs
- Further **improved the surface chemistry using a synthetic biopolymer coating.**
- For example, the **water-soluble QDs can be stabilized with a positively charged polymer or a layer of chemically denatured bovine serum albumin (BSA).**
- A key finding is that the **polymer coating restores the optical properties of QDs** nearly to that of the original QDs in chloroform. The polymer layer **also provides functional groups (amines and carboxylic acids) for covalent conjugation** with a variety of biological molecules.

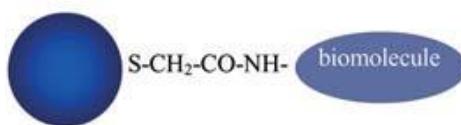
Methods

- A similar approach, in which **engineered proteins with a linear positively-charged peptide are directly adsorbed onto negatively charge nanocrystals** through electrostatic interactions
- Most recently, Wu and coworkers used an **amine-modified polyacrylic acid polymer to coat the surface of QDs**. The modified polymer was no longer soluble in water, and strongly adsorbed onto TOPO-capped QDs via hydrophobic interactions in chloroform. An important feature of this procedure is that **QDs are solubilized without removing the surface ligands (TOPO), which maintains the optical properties of QDs in an aqueous environment.**
- Similarly, Bubertret et al. **encapsulated hydrophobic QDs in small micelles** and demonstrated their use in **in-vivo cellular imaging**. QDs have been used for **multiplexed optical encoding and high-throughput analysis** of genes and proteins

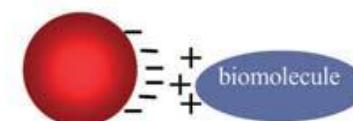
Methods

- Polystyrene beads are embedded with multicolor CdSe QDs at various color and intensity combinations. The use of six colors and ten intensity levels can theoretically encode one million protein or nucleic acid sequences. Specific capturing molecules such as peptides, proteins, and oligonucleotides are covalently linked to the beads and are encoded by the bead's spectroscopic signature. A single light source is sufficient for reading all the QD-encoded beads.
- To determine whether an unknown analyte is captured or not, conventional assay methodologies (similar to direct or sandwich immunoassay) can be applied. This so-called “bar-coding technology” can be used for gene profiling and high-throughput drug and disease screening.
- Based on different principles, Natan and coworkers reported a metallic nanobarcoding technology for multiplexed bioassays.
- Together with QD-encoded beads, these “barcoding” technologies offer significant advantages over planar chip devices (e. g., improved binding kinetics and dynamic range), and are likely to find use in various biotechnological applications.

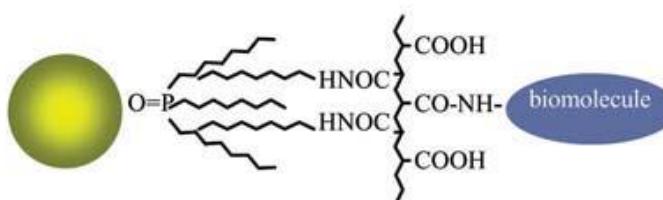
(a) Bifunctional linkage



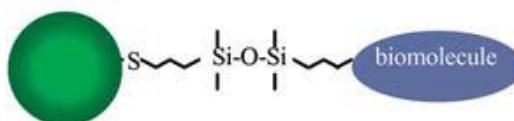
(d) Electrostatic Attraction



(b) Hydrophobic Attraction



(c) Silanization



(e) Encoded Beads

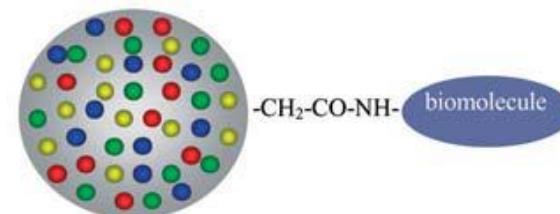


Figure: Schematic illustration of surface modification methods for linking quantum dots to biomolecules.

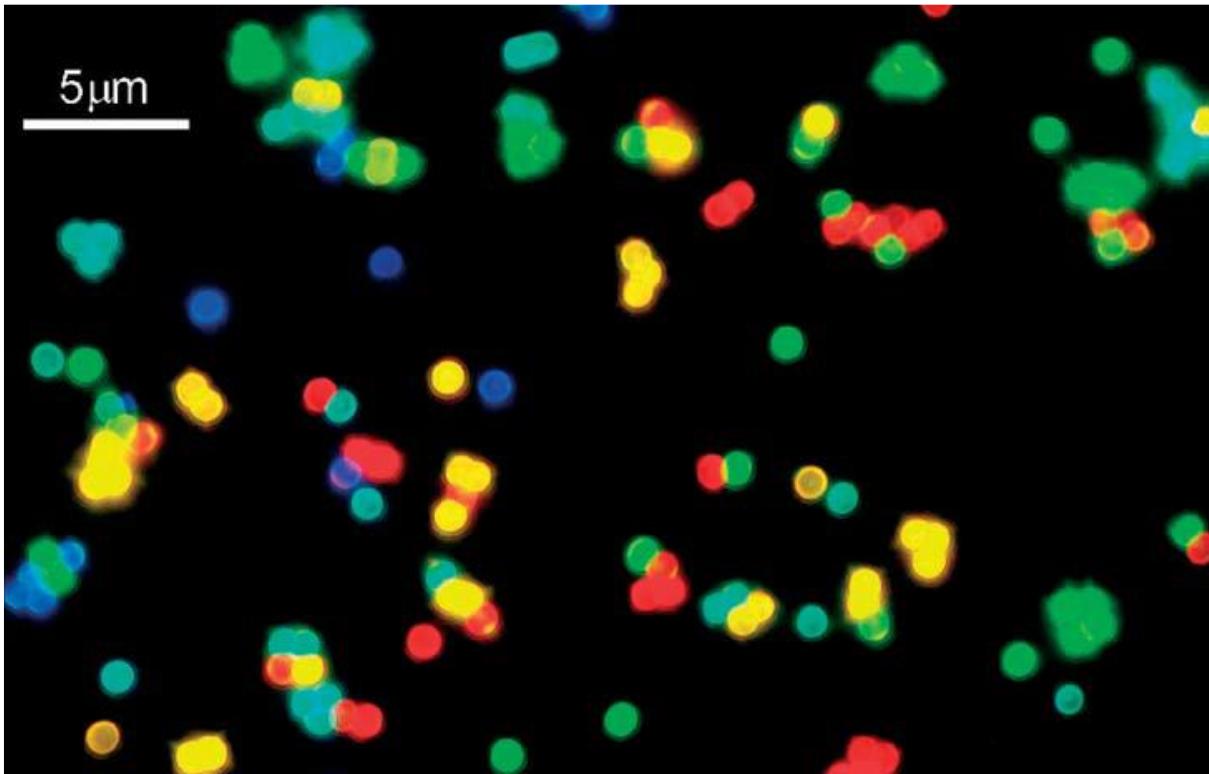


Figure : Fluorescence micrograph of a mixture of CdSe/ZnS QD-tagged beads emitting single-color signals at 484, 508, 547, 575, and 611 nm. The beads were spread and immobilized on a polylysine-coated glass slide, which caused a slight clustering effect.

Outlook

- A number of biological labeling applications have been demonstrated for QDs, including **DNA hybridization, immunoassays, and receptor-mediated endocytosis**.
- In particular, multicolor quantum dots are well-suited for the simultaneous labeling of multiple antigens on the surface of normal and diseased cells.
- The high photostability of QDs allows not only **real-time monitoring or tracking of intracellular processes over long periods of time**, but also **quantitative measurements of fluorescent intensity**.
- They allow **target detection at the single-copy level**, and provide detailed structure information of biological specimens.
- **Far-red and near-infrared QDs are well-suited for applications in in-vivo molecular imaging** and ultrasensitive biomarker detection.
- **Visible light has been used for cellular imaging and tissue diagnosis**, but optical imaging of deeper tissues (millimeters) requires the use of **far-red or near-infrared light in the spectral range of 650–900 nm**. This wavelength range provides a “clear” window for in-vivo optical imaging **because it is separated from the major absorption peaks of blood and water**

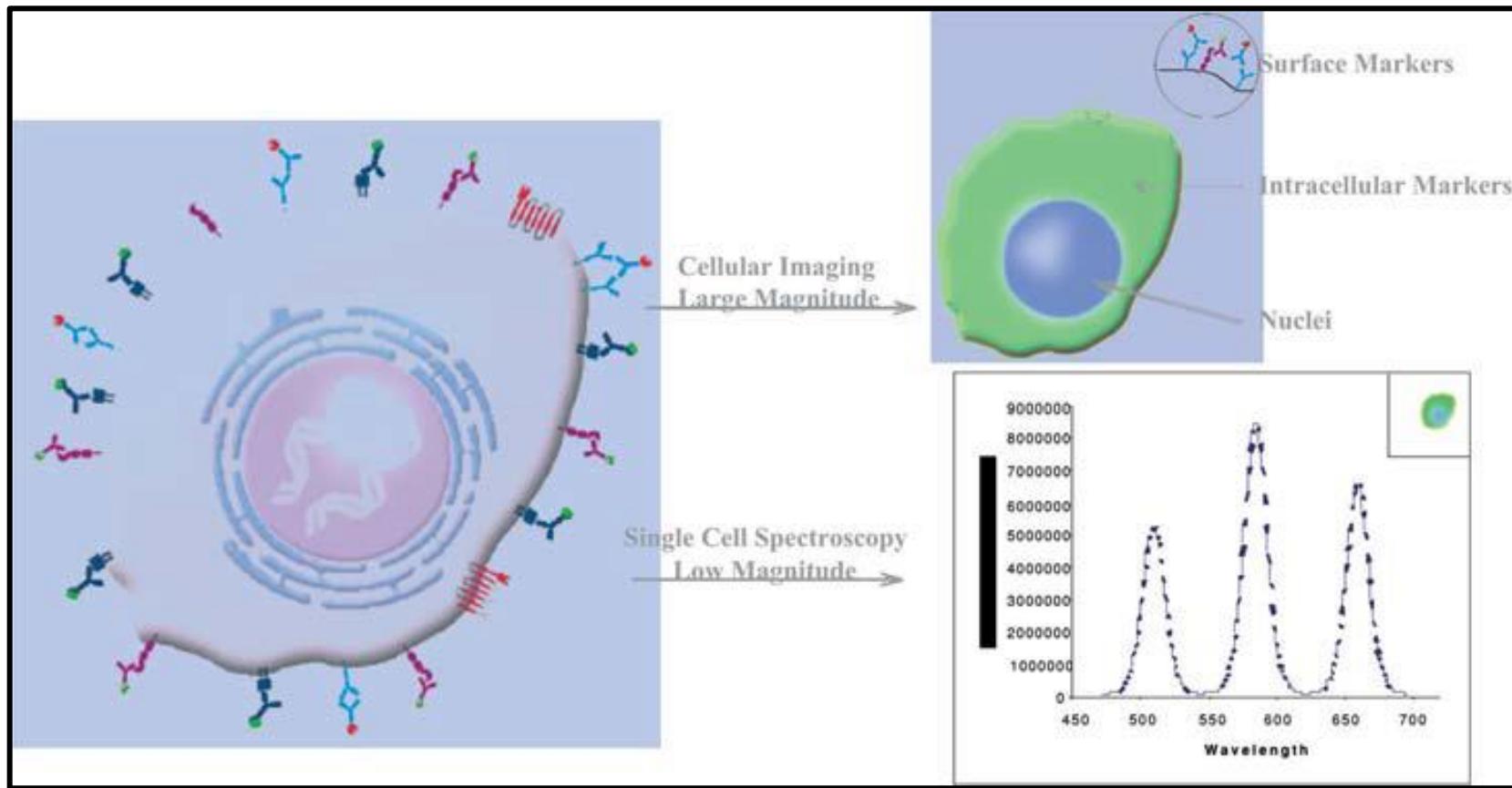


Figure : Schematic illustration of cell staining using biomolecules attached with multicolor QDs.
Small color particles represent bioconjugated quantum dots.

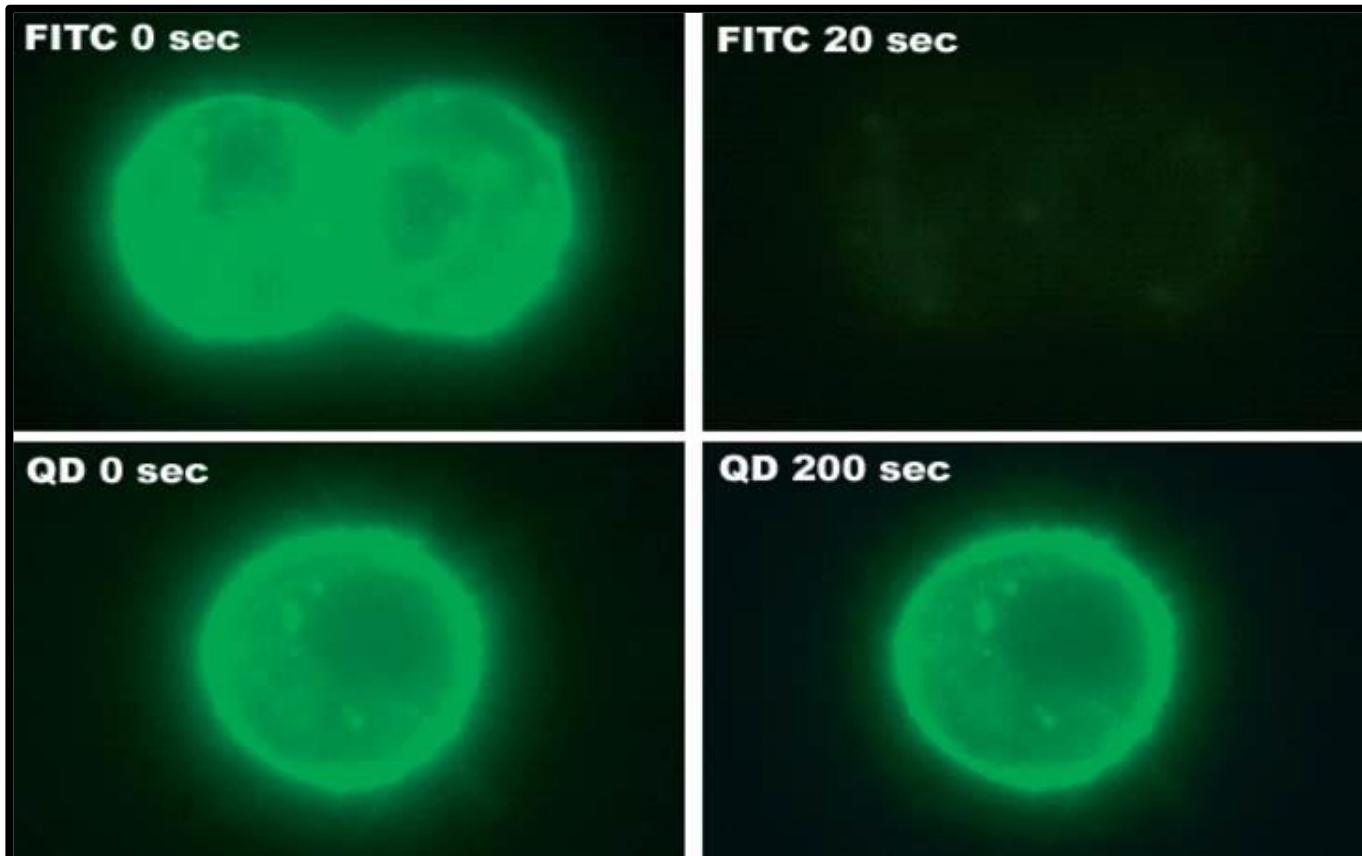


Figure: Immunofluorescence images of **human breast tumor cells (BT-474)** stained with **organic dye (fluorescein isothiocyanate; FITC)** and **green quantum dots**. Within only 20 seconds of illumination, the organic dye was almost completely photobleached (top panel), while the QD fluorescence image was stable

Outlook

- Under photon-limited *in-vivo* conditions (where light intensities are severely attenuated by scattering and absorption), the large absorption coefficients of QDs (on the order of $10^6 \text{ cm}^{-1}\text{M}^{-1}$, which is 10–100 times larger than those of common organic dyes) will be essential for efficient probe excitation.
- Unlike current single-color molecular imaging, multi-wavelength optical imaging with QDs will allow intensity ratioing, spatial colocalization, and quantitative target measurements at single metastasized tumor sites and for single anatomical structures.
- With an inert layer of surface coating, the nanocrystals are less toxic than organic dyes

Outlook

- In preliminary studies, luminescent QDs were **conjugated to transferrin** (an iron-transport protein), **to antibodies that recognize cancer biomarkers**, and **to folic acid** (a small vitamin molecule which is recognized by many cancer cells).
- In each case, it was found **that receptor-mediated endocytosis occurred and the nanocrystals were transported into the cell**. Single QDs as well as clusters of dots **trapped in vesicles were clearly visible inside living cells**.
- In conclusion, semiconductor QDs have been developed as **a new class of biological labels with unique advantages and applications** that are not possible with organic dyes or fluorescent proteins.
- When **conjugated with fully functional biomolecules** such as peptides, protein, and oligonucleotides, this class of fluorescent tags is **well-suited for ultrasensitive imaging and detection**.
- It is envisioned that the design and construction of multifunctional QDs will allow **molecular imaging and diagnostics of single diseased cells**.