Particulate carrier systems for mucosal DNA vaccine delivery

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Why mucosal?
``Improvements that make vaccine delivery easier and safer, decrease dependency on the cold chain or reduce number of immunization interventions needed, could have a significant impact...``

Friede & Aguado, ADDR 57 (2005) 325-331
Initiative for Vaccine Research, WHO

‘Ideal’ vaccine: the SAFE concept

S table under high temperature and freezing conditions

A ffordable, allowing large scale vaccination campaigns in developing countries

F ast: single-shot (pulsatile release?) increasing compliance, coverage of certain age groups (i.e. adolescents)

E asy application (nasal, topical, oral, pulmonary), avoiding parenteral administration and risk of infection
Disease burden in developing countries caused by unsafe injections

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
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<tbody>
<tr>
<td>Hep B</td>
<td>21.7 M</td>
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<tr>
<td>Hep C</td>
<td>2 M</td>
<td>42%</td>
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<tr>
<td>HIV*</td>
<td>96 k</td>
<td>2%</td>
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</table>

*worldwide

WHO/BHT/DCT/01.3, pp. 1-7

HIV infection changing paradigm: a ‘tale of two infections’

Picker & Watkins, Nat Immunol 6 (2005) 430
Mucosal surfaces are the port-of-entry for infectious diseases

Role of Mucosal T-cells in HIV:

- HIV/SIV infect mucosal CD4+CCR5+ T-cells
- rapid depletion by lytic viral replication
- viral reservoirs in resting memory T-cells
- functional and structural degradation of mucosal tissue
- increased antigen exposure leads to opportunistic infections (OIs)
- OIs trigger activation of CD4+CCR5+ T-cells

1st [mucosal] line of defense:
Present and Future

Picker & Watkins, Nat Immunol 6 (2005) 430
Haase Nat Rev Immunol 5 (2005) 783
“There has been minimal global effort for clinical trial assessment of vaccine approaches that have the potential to protect at mucosal surfaces during early events…”

“…strategies are needed that could elicit mucosal immune responses in addition to systemic immune responses…”

EU Strategic Position on HIV Vaccine Development, Vaccine 2005, in press

Pulmonary Immunity
Bronchial Associated Lymphoid Tissue (BALT):

- BALT is *not* a constitutive structure of the healthy adult lung.

- Induced by high antigen load, infection, inflammation.

- Sampling from lumen by epithelial cells, not through lymph system.

- Formed independently of lymphotoxin α (Ltα), inducer of 2° lymphoid organs in embryogenesis and modulator of immune response.
Respiratory immunity in the absence of lymphoid structures: iBALT

- Lymphotoxin (LT) α−/− lack lymph nodes and PP, show disrupted spleen and NALT
- LTα KO mice form lymphoid structures de novo in the lung on influenza challenge
- Formation suggested to be mediated by epithelial cells, affecting Mo, DC, T-cells, etc.
- “iBALT” structures are capable of staging adaptive immune response on 2° infection

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Effector Lymphoid Tissue (ELT)

- DC migration & presentation
- T_{eff}: effector cells  T_{em}: effector memory cells  T_{cm}: central memory cells

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van Panhuys, Trends Immunol 26 (2005) 242
ELT paradigm:

- Defines and includes pool of $T_{em}/T_{eff}$ cells outside 2° lymphoid tissue.
- Formation is the result of stable retention of T-cells post AG stimulation.
- $T_{eff}$ and $T_{em}$ cells stably localized at port-of-pathogen-entry for fast reaction to 2° infection.
- Not limited to mucosal tissues, includes all organs exposed to pathogens.
- Not encapsulated, no anatomically or histologically defined structures.

Questions:

- Which cells, mediators, receptors play important role in ELT formation?
- How is selective recruitment, retention, long-term survival and replenishment of $T_{em}/T_{eff}$ cells regulated?
- Orchestration of immune response between ELT and 2° lymphoid tissue on 2° infection?
- Optimal vaccine/mucosal delivery system? Adjuvant? Targeting?
Pulmonary vaccination: Tuberculosis

Pulmonary delivery of a TB vaccine

Advantages

- Immunity at primary infection site
- Mucosal and systemic immunity
- Reduced need for medical staff
- Non-invasive
**Tuberculosis**
- 2.2 million deaths per year
- 2 billion infected
- 8 million new cases per year
- 10-15 individuals annually infected by single untreated patient
- BCG is not a satisfactory vaccine
- No vaccine available for HIV patients more exposed to active TB
- Drug regimens are complicated, poor compliance, development of resistant strains
- MDR-TB rising, therapy is expensive

*GPEN 2006*

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**M. tuberculosis, HIV have an intracellular lifestyle**
Example: the *M. tuberculosis* genome

- 4.411 Mbp, 90.8% protein coding genes
- Genes with attributed functions: 2,441, unknown: 606
- Specific open reading frames (ORF) absent from *M. bovis*: 129.
- Absent ORF represent information for potential antigens to be integrated in novel pDNA vaccines against tuberculosis.
DNA Vaccines for Tuberculosis


- Other plasmids encoding proteins related to different stages of *M. tuberculosis* development.

Optimisation of DNA vaccines - increasing cellular/humoral responses by:

- immunostimulatory sequences neighbouring CpG motifs: pupuCGpypy (pu: A,G; py: T, C)

- integration of genetic information for cytokines:
  - Th1 cytokines (IL-12, IFN-γ) to stimulate cytotoxic T-cell (CTL) response
  - Th2 cytokines (IL-4, -5, -10) to stimulate humoral response
DNA vaccines: formulation parameters

- DNA vaccine parameters: polyepitope, size, enzyme stability
- Nature pathogen/disease: viral/bacterial, route of entry, progression of disease
- Desired immune response: Humoral, CTL, Th1/Th2
- Delivery system: Administration route, targeting, delivery device

DNA vaccines: administration routes alternative to injection

1) mucosal: oral, nasal, vaginal, rectal, pulmonary
   - interaction with local immunoactive tissues, e.g. Peyer’s patches
   - induction of both, local and systemic immune response (i.e., IgA and IgG)
   - cross-talk between mucosal tissues (Mucosal Associated Lymphoid Tissues, MALT)
   - strong involvement of dendritic cells (DC), especially in the lung

2) Gene gun
   - intradermal injection of DNA vaccine coated gold particles
   - stronger Th2 bias than i.m. injection
Gene gun approach:

- DNA coated particles are injected into the cells: improvement of uptake by Langerhans’ cells
- less priming by CpG motifs through TIR interaction
- lower expression of CD, MHC
- resulting in Th2 bias

Aims:
1. In vitro testing Calu-3, DC
2. Evaluate T-cell response
3. Compare i.m. to pulmonary application
4. Explore the effect of carrier system

Concept

New DNA construct  
Class I specific epitopes

Class I transgenic mouse model

Chitosan nanoparticles

Pulmonary aerosol delivery

Aims:
1. In vitro testing Calu-3, DC
2. Evaluate T-cell response
3. Compare i.m. to pulmonary application
4. Explore the effect of carrier system
Polymer-based DNA vaccine delivery systems

- condensation of DNA by electrostatic interactions
- reduction in size, zetapotential
- protection against enzymatic degradation, DNase I/II
- endolysosomal escape
- stability, shelf-life
- toxicity

Chitosan nanoparticles

- Chitosan n.p. were proven to be efficient carriers for oral delivery of DNA vaccine against peanut allergy (Leong et al.)

- Chitosan-DNA complexes (nano-size) showed good pulmonary transfection in-vivo (Köping-Höggård et al.)

- Chitosan-DNA complexes (nano-size) were shown to be safe and efficient gene delivery systems in epithelial cells (Thanou et al.)
Preparation of chitosan nanoparticles

Chitosan solution
GCN2006
DNA solution in Na_2SO_4
55°C Vortex Nanoparticles formation

Characterization of size, zetapotential, DNase protection, DNA loading and release

Loading Efficiency

chitoplex suspension
free DNA in supernatant
PicoGreen binds to free DNA
fluorescence

Loading Efficiency (LE) = \frac{(total \ DNA - free \ DNA)}{total \ DNA} \times 100 \%

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Characteristics of chitoplexes

- Size of chitoplexes: 200 - 400 nm
- Charge at pH 5.5: 20 - 27 mV
  - strongly dependent on pH
  - positive charge good for cell attachment and uptake
- Loading Efficiency: > 95%
  - efficient procedure; no material loss

→ Size, zetapotential and LE independent of (N/P) ratio
→ Strong charge interactions

Enzymatic assays

Is DNA in chitoplexes protected against nucleic acid degradation by chitosan?
→ Incubation with DNase I

When the chitosan in chitoplexes is degraded by enzymes, is the DNA released in intact form?
→ Incubation with chitosanase
Incubation with DNase I (1)

Free DNA → Fragmented DNA

Incubation with DNase, 37°C → Intact DNA?

Analysis by agarose gel electrophoresis

- 55 min, 100 V

Incubation with DNase I (2)

Naked DNA → cleaved plasmid, supercoiled plasmid

pRSV → marker

Ratio (N/P) 2:1 → 10 20 0 10 20 40

Ratio (N/P) 3:1 → 0 10 20 40

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Conclusion: Incubation with DNase I

- Compared with naked DNA, the DNA in chitoplexes is protected against nucleic acid degradation by chitosan.
- The more chitosan, the more protection?
  - Ratio (N/P) 2:1 is less protected
  - no significant differences at ratios between (N/P) 3:1 and 6:1
Incubation with chitosanase (1)

- Chitosanase present in micro-organisms and plants
- used chitosanase: from *Streptomyces griseus*

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\[\text{degradation products: oligochitosan 2 - 6}\]
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Stop solution: 1M KOH
In humans: degradation by lysozyme

Incubation with chitosanase (2)

- Free DNA
- chitoplexes

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\text{Extraction with phenol: chloroform: isoamyl alcohol (25:24:1)}
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DNA in aqueous phase

Analysis by agarose gel electrophoresis

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\text{55 min, 100 V}
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Conclusions: chitosanase assay

Chitoplexes are partly degraded by chitosanase.

Only free DNA, no chitoplexes, are extracted.
After extraction some free DNA stays at loading position.

Free DNA is released and partially fragmented.
Fragmentation is due to the stop solution (1M KOH).
After enzymatic degradation of chitoplexes, DNA is intactly released.

DNA vaccines: advantages

Immunogenicity  induces humoral and cellular immune responses
low effective dosage in animal models

Safety  unable to revert into virulence,
no toxic treatment needed as in live vaccines

Engineering  vectors easy to manipulate, fast testing
combinatorial approaches easily adapted

Manufacture  low costs, reproducible large-scale production

Stability  temperature-stable than conventional vaccines
long shelf-life

Mobility  easy storage and transport, no cold chain
DNA vaccines: Challenges

- adequate animal models
- extension of plasmid survival: better immune response?
- will prolongation of antigen synthesis elicit autoimmune responses?
- interindividual differences in immune responses?
- dendritic cell targeting
- selection of antigens -> genomics approach (inverse vaccinology)
- prime/boost regimens and adjuvants

The DNA plasmid

- ThyA
- 85A
- ESAT6
- RpoB
- 85B
- PstA1
- HSP70
- 19kD
In vitro testing

Dendritic cells

Immature DC
Good Phagocyte
Bad APC

Mature DC
Stimulation
CD80/86
CD83
MHC
Bad Phagocyte
Good APC
Dendritic cells maturation

Comparison of cell culture models of the airway epithelium

<table>
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<th>RTC</th>
<th>Calu-3</th>
<th>16HBE14o-</th>
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<td>2.5x10&lt;sup&gt;7&lt;/sup&gt;</td>
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</table>

RTC: rabbit tracheal epithelial cells, Calu-3: human submucosal gland cell line, 16HBE14o-: human bronchial cell line, P<sub>app</sub>: apparent permeability (10<sup>-7</sup>cm s<sup>-1</sup>), CFTR: Cystic Fibrosis Transmembrane Regulator protein, P-gp: P-glycoprotein

# = own data, all other data taken from current literature.
Calu-3 cells: mucus staining Periodic Schiff’s, Alcian Blue

- Calu-3 express human MUC1, MUC4, MUC5 and MUC5B genes
- Calu-3 secrete proteoglycans and sulfated mucins
- Calu-3 apical surface fluid exerts anti-bacterial activity
- Calu-3 are used for investigation of mucus as a barrier to gene delivery

Meaney et al., Cell culture models of biological barriers, Harwood 2002

Uptake by human bronchial epithelial cells (Calu-3) in vitro

LAMP-1  rhodamine-DNA  superimposition

In vivo testing

MHC class I

The 3D structure of a class I MHC molecule
Experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>DNA plasmid</th>
<th>Application</th>
<th>Formulation</th>
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<td>I</td>
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<tr>
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<tr>
<td>IV</td>
<td>Polyepitope</td>
<td>Endotracheal</td>
<td>Chitosan n.p.</td>
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Immunization Regimen

- DNA polyepitope 25µg
- protein boost 20µg
- sacrifice

Week 1<br>Week 4<br>Week 7<br>Week 9 + 10 days
**Figure 1: IFN-γ (M. tuberculosis sonicate)**

![Graph showing IFN-γ levels with different treatments](image1)

**Figure 2: IFN-γ (19kD protein)**

![Graph showing IFN-γ levels with different treatments](image2)
Conclusions

Maturation of DCs in culture

Induced in-vivo T cell responses toward M. tuberculosis sonicate.

The pulmonary (e.t.) immunization had a significant advantage over i.m. administration.

Chitosan n.p. enhanced IFN-g production in comparison to the DNA solution.
Conclusions:

Mucosal surfaces of the lung are suitable for eliciting local and systemic immune response.

Problems of parenterally applied vaccines are avoided (patient compliance, risk of infection, infrastructure).

DNA-vaccine offer advantages over subunit vaccines (combination of antigenic structures and adjuvants, stability).

Optimization of both vaccines and carrier systems for mucosal application, especially if applied pulmonary, is necessary.