PREPARACIÓ DE PARTÍCULES GEL D´ADN COM A SISTEMES D´ALLIBERAMENT CONTROLAT

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Departament de Fisiologia-Facultat de Farmàcia
Gene delivery

- Degradation on the bloodstream by the endogenous nucleases
- Is too large to cross the cellular membranes
Encapsulation

- TO CONCENTRATE ENCAPSULATED MATERIAL
- TO PROTECT ENCAPSULATED MATERIAL
- TO RELEASE ENCAPSULATED MATERIAL IN A CONTROLLED WAY
OPPOSITELY CHARGED POLYELECTROLYTES (OR SURFACTANTS AND POLYELECTROLYTES)

ASSOCIATIVE PHASE SEPARATION

VISCOUS LIQUID
GEL, LIQUID CRYSTAL
OR PRECIPITATE
**Aims**

- **Encapsulation**
- **Associative phase separation**
- **Gelation formed at WATER/WATER emulsion type interfaces**
High content DNA reservoir

Without adding any kind of cross-linker or organic solvent
How To Prepare DNA Gel Particles

1. Drop formation and addition
2. Incubation

DNA gel
Cationic polyelectrolyte
DNA gel particles
# Systems

<table>
<thead>
<tr>
<th>CATIONIC AGENT</th>
<th>dsDNA</th>
<th>ssDNA</th>
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<td><strong>SINGLE-TAIL SURFACTANTS</strong></td>
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<td>DTAX</td>
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<td><strong>REVIEW</strong></td>
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<td>Soft Matter, 6, 3143-3156 (2010)</td>
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</table>
Surfactant-DNA Gel Particles

Phase map: ds-DNA (Salmon testes)-CTAB System

![Graph showing the phase map of ds-DNA (Salmon testes)-CTAB System with markers for Charge Equilibration at 1 Φ and 2 Φ.]
Protein-DNA Gel Particles

PROTEIN-POLYELECTROLYTE COMPLEX

INTERNAL POLYELECTROLYTE (DNA)

LYSOZYME from chicken egg white
Polysaccharide-DNA Gel Particles

POLYELECTROLYTE-POLYELECTROLYTE COMPLEX

INTERNAL POLYELECTROLYTE (DNA)

CHITOSAN

\[ R = \frac{[\text{ds-DNA}]}{[\text{CH}^+]}} \]

R = 25.0, R = 13.3, R = 2.6
PHYSICOCHEMICAL CHARACTERIZATION

- Degree of DNA Entrapment
- Particle Morphology
- Secondary Structure of DNA
- Swelling / Dissolution Behaviour
- Kinetics of DNA Release
- Degree of Complexation

In vitro BIOCOMPATIBILITY

- Haemocompatibility
- Cytotoxicity
Loading Efficiency (%) = \frac{[\text{total amount of DNA} - \text{non-bound DNA}]}{\text{total amount of DNA}} \times 100
LOADING CAPACITY(%) = \[\frac{\text{total amount of DNA} - \text{(non-bound DNA)}}{\text{weight of particles}}\] x 100

Degree of DNA Entrapment

Spectrophotometric determination OD\text{260}

+1 16
+1 12
+2 12
+1 12
+1 12
Degree of DNA Entrapment

Spectrophotometric determination $\text{OD}_{260}$

LOADING CAPACITY(%) = \[\frac{\text{(total amount of DNA)} - \text{(non-bound DNA)}}{\text{weight of particles}}\] x 100

- **LS**
  - 14.1 KDa
  - +9

- **PS**
  - 5.1 KDa
  - +21
Degree of DNA Entrapment

Spectrophotometric determination $OD_{260}$

$$LOADING\ CAPACITY(\%) = \frac{[\text{total amount of DNA} - \text{non-bound DNA}]}{\text{weight of particles}} \times 100$$
DNA distribution

Spectrophotometric determination OD$_{260}$

DNA COMPLEXED (%)
DNA distribution
Spectrophotometric determination $\text{OD}_{260}$

DNA COMPLEXED (%)
DNA distribution

Spectrophotometric determination $\text{OD}_{260}$

DNA COMPLEXED (%)

![Bar chart showing DNA complexed percentages for various samples, including CTAB, DTAB, DTAC, DTAF, ALA, IAM, DDAB, LS, LSPS15, LSPS30, LSPS50, LSPS70, LSPS85, PS, CHIT50, CHIT150, and CHIT400.]

Magnetically stirred

CORE-SHELL PARTICLES
SOLID PARTICLES
DNA distribution

Spectrophotometric determination $\text{OD}_{260}$

DNA COMPLEXED (%)

CTAB

ALA

Magnetically stirred
### Secondary Structure of DNA

#### Fluorescence Microscopy

**SURFACTANT SYSTEMS**

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>dsDNA Structure</th>
<th>ssDNA Structure</th>
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<tbody>
<tr>
<td>CTAB-dsDNA</td>
<td>16 (+1)</td>
<td>16 (+1)</td>
</tr>
<tr>
<td>DTAB-dsDNA</td>
<td>12 (+1)</td>
<td>12 (+1)</td>
</tr>
<tr>
<td>ALA-dsDNA</td>
<td>12 (+2)</td>
<td>12 (+2)</td>
</tr>
<tr>
<td>LAM-dsDNA</td>
<td>12 (+1)</td>
<td>12 (+1)</td>
</tr>
<tr>
<td>DDAB-dsDNA</td>
<td>12 12 (+1)</td>
<td>12 12 (+1)</td>
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<th>Surfactant</th>
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</tr>
<tr>
<td>DTAB-ssDNA</td>
<td>12 (+1)</td>
<td>12 (+1)</td>
</tr>
<tr>
<td>ALA-ssDNA</td>
<td>12 (+2)</td>
<td>12 (+2)</td>
</tr>
<tr>
<td>LAM-ssDNA</td>
<td>12 (+1)</td>
<td>12 (+1)</td>
</tr>
<tr>
<td>DDAB-ssDNA</td>
<td>12 12 (+1)</td>
<td>12 12 (+1)</td>
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</table>

**DNA complexed (%)**

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>DNA Complexed (%)</th>
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<tbody>
<tr>
<td></td>
<td>79 ± 10</td>
</tr>
<tr>
<td></td>
<td>94 ± 1.2</td>
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<td>99 ± 0.2</td>
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<tr>
<td></td>
<td>99 ± 0.3</td>
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<tr>
<td></td>
<td>97 ± 1.5</td>
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</table>

**Emission Wavelengths:**

- AO-dsDNA: 530 nm
- AO-ssDNA: 640 nm
Particle Morphology

Scanning Electron Microscopy

• SURFACTANT SYSTEMS

Outer

- CTAB-dsDNA 16 (+1)
- CTAB-ssDNA 16 (+1)
- DTAB-dsDNA 12 (+1)
- DTAB-ssDNA 12 (+1)

Inner

DNA complexed (%)

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<tr>
<td>CTAB-dsDNA</td>
<td>52 ± 10</td>
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<tr>
<td>CTAB-ssDNA</td>
<td>70 ± 12</td>
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<tr>
<td>DTAB-dsDNA</td>
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<td>99 ± 0.3</td>
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<tr>
<td>DTAB-ssDNA</td>
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<td>94 ± 1.2</td>
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</table>
Particle Morphology

Scanning Electron Microscopy

• SURFACTANT SYSTEMS

Outer

DDAB-dsDNA
12 12 (+1)

DDAB-ssDNA
12 12 (+1)
NR-hydrophobic lipid: 657 nm
Particle Morphology

Scanning Electron Microscopy

**SURFACTANT SYSTEMS**

Outer

DDAB-dsDNA

\[12 \ 12 \ (+1)\]

DDAB-ssDNA

\[12 \ 12 \ (+1)\]

AO-dsDNA: 530 nm

AO-ssDNA: 640 nm
Secondary Structure of DNA

Fluorescence Microscopy

• PROTEIN SYSTEMS

AO-dsDNA: 530 nm
AO-ssDNA: 640 nm

Increasing PS content

LS-dsDNA
LSPS15-dsDNA
LSPS30-dsDNA
LSPS50-dsDNA
LSPS70-dsDNA
LSPS85-dsDNA
PS-dsDNA
Secundary Structure of DNA

Fluorescence Microscopy

• POLYSACCHARIDE SYSTEMS

$R = \frac{[\text{ds-DNA}]}{[\text{CHIT}]}$ with [ds-DNA] equal at 60 mM

AO-dsDNA: 530 nm
AO-ssDNA: 640 nm

Increasing CHIT content

$R: 25$  $R: 20$  $R: 12$  $R: 3$
In vitro Kinetics

PARTICLES

SUPERNATANTS

Rw > 1
SWELLING

Rw = 1

Rw > 1
DISSOLUTION
Swelling and Dissolution Behaviour

Relative weight (\(RW=W_t/W_i\))
DNA Release From Particles

Spectrophotometric determination $\text{OD}_{260}$

**SURFACTANT-DNA SYSTEMS**

- CTAB-dsDNA
- CTAB-ssDNA
- DTAB-dsDNA
- DTAB-ssDNA
- LAM-dsDNA
- LAM-ssDNA
- ALA-dsDNA
- ALA-ssDNA
- DDAB-dsDNA
- DDAB-ssDNA

cumulative DNA release (%)

time (h)
Swelling and Dissolution Behaviour

Relative weight \((RW=\frac{W_t}{W_i})\)
DNA Release From Particles

Spectrophotometric determination $\text{OD}_{260}$
Swelling and Dissolution Behaviour

Relative weight \((RW=W_t/W_i)\)
DNA Release From Particles

Spectrophotometric determination $\text{OD}_{260}$

![Graph showing DNA release from polysaccharide-DNA systems. The graph plots cumulative DNA release (%) against time (h) for different systems labeled as CHIT50-dsDNA, CHIT150-dsDNA, and CHIT400-dsDNA.]}
I. Haemocompatibility: Surfactant-DNA gel particles

- HYDROPHOBIC CHAIN LENGTH ✓
- NUMBER OF CHARGES ✓
- COUNTERION INFLUENCE ?

Haemolysis assay

RED BLOOD CELLS

SURFACTANT SOLUTIONS

DNA GEL PARTICLES

1: Solutions or DNA gel particles

2: PBS buffer

3: RBC dispersion

INCUBATION
room temperature
10 - 360 min

CENTRIFUGATION
10,000 rpm 5 min
Haemolysis assay

- Red blood cells
- Surfactant solutions
- DNA gel particles

0% haemolysis
Isotonic PBS buffer

100% haemolysis
Bidistilled water

Centrifugation
10,000 rpm 5 min

OD: 540 nm

0% ➔ 20% ➔ 50% ➔ 100%
Haemolysis in solution

Spectrophotometric determination $\text{OD}_{540}$

<table>
<thead>
<tr>
<th>System</th>
<th>DTAB (µg/mL)</th>
<th>DTAC (µg/mL)</th>
<th>DTATf (µg/mL)</th>
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<tr>
<td>HC$_{50}$</td>
<td>510</td>
<td>443</td>
<td>468</td>
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Haemolysis induced by surfactants in DTA-DNA gel particles

- STRONGLY DEPENDENT OF THE SURFACTANT-DNA INTERACTION
- FAVOURED IN THE CASE OF ss-DNA DUE TO ITS AMPHIPHILIC CHARACTER
Haemolysis vs. DNA Release

ds-DNA released = 100 µg/mL
- DTAB: 80%
- DTAC: 60%
- DTATf: 30%

ss-DNA released = 6 µg/mL
- DTAB: 70%
- DTAC: 50%
- DTATf: 20%

DTATf < DTAC < DTAB
Degree of counterion dissociation ($\alpha$)

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>DTAB</th>
<th>DTAC</th>
<th>DTATf</th>
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<td>$\alpha$ values</td>
<td>0.26$^1$</td>
<td>0.37$^2$</td>
<td>0.13-0.15$^3$</td>
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$\alpha$ content (µg/mL): 1030 | 830 | 490 |

Haemolysis: DTAB > DTAC > DTATf

II. Cytotoxicity: Mixed-Protein DNA gel particles

- Biocompatible
- Protective
- Biodegradable
- Controlled release
- Long-acting
- Reproducible

Protein-DNA systems:
- LSPS15-dsDNA
- LSPS50-dsDNA
- LSPS85-dsDNA
- LS-dsDNA
- LSPS30-dsDNA
- LSPS70-dsDNA
- PS-dsDNA

Increasing PS content

Morán et al., *Macromolecular Bioscience* (2012)
Cytotoxicity in vitro

Day 1
Culture preparation

Formation of the cell monolayer

75cm² culture flasks

80% confluence

3T3 FIBROBLASTS

Trypsinization of adherent cells
trypsin/EDTA

Cell counting: EB/AO

Cellular suspension

1 x 10⁵ cell/mL (3T3)
5 x 10⁴ cell/mL (HeLa) (100 µl/well)

37 °C
5% CO₂
24 h

HeLa
HUMAN EPITHELIAL CARCINOMA
**Cytotoxicity in vitro**

**Day 2 Assay**

**Incubation of the cells in the presence of DNA gel particles**

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<td>A</td>
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<td>C</td>
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<td>E</td>
<td>F</td>
<td>G</td>
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C: CONTROL (100 µl/well)
S: SOLUTIONS (100 µl/well)
P: PARTICLES (100 µl/well + 1p)

37 ºC
5% CO₂
24 h
**Cytotoxicity in vitro**

**Day 3**

End-point measurements

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**Cell death/viability measurements**

- 37 ºC
- 5% CO₂
- 3 h

**DMSO agitation**

- 10 min

**OD: 550 nm**

**% CELL VIABILITY**
Cytotoxicity *in vitro*: Proteins in solution

3T3 fibroblasts

HeLa human epithelial carcinoma

IC$_{50}$ (LS) > 2000 µg/mL

IC$_{50}$ (PS) = 140-250 µg/mL
Cytotoxicity *in vitro* induced by protein-DNA gel particles at millimeter scale

\[ R = \frac{[ds-DNA]}{[P+]} = 1 \] with \([ds-DNA]\) and \([P+]\) equal at 60 mM

1 PARTICLE FOR WELL PLATE

t_{\text{incubation}} = 24 \text{ h}

PROTEIN SOLUTION

* Significant differences (p<0.05) from the corresponding protein solution
Gene delivery: Endocytosis

R=[ds-DNA]/[P+]=1 with [ds-DNA] and [P+] equal at 2.5 mM

<table>
<thead>
<tr>
<th>System</th>
<th>Mean size (nm)</th>
<th>P. I.</th>
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<tbody>
<tr>
<td>CTAB-dsDNA</td>
<td>139 ± 8</td>
<td>0.456 ± 0.01</td>
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<tr>
<td>CTAB-ssDNA</td>
<td>126 ± 1</td>
<td>0.403 ± 0.01</td>
</tr>
<tr>
<td>PS-dsDNA</td>
<td>387 ± 8</td>
<td>0.782 ± 0.30</td>
</tr>
<tr>
<td>PS-ssDNA</td>
<td>343 ± 10</td>
<td>0.140 ± 0.03</td>
</tr>
<tr>
<td>LS-dsDNA</td>
<td>9208 ± 2800</td>
<td>0.313 ± 0.40</td>
</tr>
<tr>
<td>LS-ssDNA</td>
<td>9450 ± 2000</td>
<td>0.781 ± 0.50</td>
</tr>
</tbody>
</table>

Cytotoxicity *in vitro* induced by protein-DNA gel particles at submicron scale

\[ R = \frac{[ds-DNA]}{[P^+]}, \text{ with } [ds-DNA] \text{ and } [P^+] \text{ equal at } 2.5 \text{ mM} \]

\[ t_{\text{incubation}} = 24 \text{ h} \]

* Significant differences (p<0.05) from the corresponding protein solution
Determination of the protein content and degree of complexation

\[ R = \frac{[\text{ds-DNA}]}{[P^+]} = 1 \] with \([\text{ds-DNA}]\) and \([P^+]\) equal at 2.5 mM

method of Bradford
Relative viabilities of tumour and non-tumour cell lines

<table>
<thead>
<tr>
<th>System</th>
<th>3T3 /IC₅₀ (mM)</th>
<th>Hela/ IC₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS-DNA</td>
<td>&lt; 0.16</td>
<td>&lt; 0.16</td>
</tr>
<tr>
<td>LSPS7.5-DNA*</td>
<td>&gt; 1.25</td>
<td>0.88</td>
</tr>
<tr>
<td>LSPS15-DNA</td>
<td>&gt; 1.25</td>
<td>0.92</td>
</tr>
<tr>
<td>LSPS30-DNA*</td>
<td>&gt; 1.25</td>
<td>0.32</td>
</tr>
<tr>
<td>LSPS50-DNA</td>
<td>&gt; 1.25</td>
<td>0.92</td>
</tr>
<tr>
<td>LSPS70-DNA</td>
<td>0.80</td>
<td>0.60</td>
</tr>
<tr>
<td>LSPS85-DNA*</td>
<td>0.75</td>
<td>0.68</td>
</tr>
<tr>
<td>PS-DNA</td>
<td>&gt; 1.25</td>
<td>&gt; 1.25</td>
</tr>
</tbody>
</table>

* Significant differences (p<0.05) between 3T3 and HeLa for the same conditions.
Internalization of protein-DNA gel particles at submicron scale

HeLa

PS-(AO/DNA) particles dispersion  \( \frac{1}{2} \) dilution (250 µL)

\( t_{\text{incubation}} = 2 \text{ h} \)

CM

FM

40x magnification
The magnitude of DNA entrapment can be controlled and controlled release systems achieved through the formation of an oppositely charged polyelectrolytes complex network giving rise to these DNA gel particles. The decrease in haemolysis and cytotoxicity as well as the formation of a releasable high DNA content reservoir renders these DNA gel particles promising DNA vehicles for use as a nonviral gene delivery system.
Acknowledgments

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MAT2012-38047-C02-01

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