Preventing Infections by Enzyme Responsive Polymeric Nanocarriers

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Outline

• Bacteriosafe project
• Miniemulsion process

• Work flow nanocapsules
• Nanocapsules characteristics
• Release of polyhexanide
• Cleavage of nanocapsules
• Stability of nanocapsules in several biological media
• Bacteria studies

• Work flow nanoparticles
• Nanoparticles characteristics
• Physico-chemical properties
• Release of octenidine
• Bacteria studies
The Bacteriosafe project

- deals with extensive burn injuries in children requiring intensive care treatment
- high incidence especially in infants under 5 years
- main focus: development of intelligent wound dressings
  - antimicrobial drug release
  - signalling of wound status (degree of inflammation/infection)

What is so special about extensive burn wounds of children?

- Large wound surface
- Problematic locations in the body
- Fluid loss, very painful
- Daily wound treatment is necessary (change of dressing)
- Frequent surgical interventions
- Bacterial infection → toxic shock syndrome (TSS) caused by exotoxins from *Staphylococcus aureus*
- Infants are at greater risk due to lack of antibodies
- Mortality of 50% if untreated
- Prophylactic antibiotics do not prevent TSS in children
How should it work?

Nanocapsules

Polyhexanide
poly(hexamethylene biguanide) hydrochloride

Hyaluronic acid, HA

Hyaluronidase

Staphylococcus aureus

Nanoparticles

Octenidine
(N-Octyl-1-[10-(4-octyliminopyridin-1-yl)decyl]pyridin-4-imin)

Fluorescent dye

poly(L-lactide), PLLA

Esterase Proteinase K
Miniemulsion process

• Heterophase system consisting of stable nanodroplets in a continuous phase
• Droplet size between 50 nm and 500 nm

**direct miniemulsion process**

- *oil-in-water-system*
- hydrophobic agent (*hexadecane*)
- surfactant (*SDS, Lutensol AT50*)

**inverse miniemulsion process**

- *water-in-oil-system*
- hydrophilic agent (*salt, sugar*)
- surfactant (*Lubrizol®U, P(E/B-b-EO]*)

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Hyaluronidase pH optimum 4.5-6.0

Staphylococcus aureus is multi resistant to antibiotics

NCs - Characteristics

Hyaluronic acid

Polyhexanide

Hydroxyethyl starch

SEM images:
(A) hyaluronic acid (HA-NCs)
(B) hyaluronic acid/polyhexanide (HA-PH-NCs)
(C) polyhexanide (PH-NCs)
(D) hydroxyethyl starch (HES-NCs)
(E) hydroxyethyl starch/polyhexanide (HES-PH-NCs)

Dynamic light scattering (DLS):
A: 350 nm
B: 320 nm
C: 260 nm
D: 230 nm
E: 260 nm
Hyaluronidase Treatment

Comparison between the fluorescence signals of hyaluronidase treated and untreated nanocapsules

Experimental setup:
500 µL NCs (10^{12} NC per mL)
500 µl hyaluronidase (32, 16, 8, 1 mg per mL)
pH: 5.5
Gently shaken at 37 °C
Incubation time: up to 23 h
Centrifugation at 4000 rpm for 20 min
Fluorescence measurements
Release of Polyhexanide

strong band at 236 nm attributed to $\pi-\pi^*$ transition of –C=N– in the biguanide

- HES-PH-NCs: no polyhexanide release (control sample)
- HA-PH-NCs:
  - 1mg/mL does not make a difference compared to HES-PH
  - 8, 16 or 32 mg/mL fairly similar amounts of PH were detected in the supernatant at specific times
Cleavage of NCs

Cleavage of hyaluronic acid leads to unsaturated fragments → absorbance at 231 nm.

Enzymes catalyze the split of glycosidic bonds between acetylglucosamine and glucoronic acid.

- HA-PH-NCs: split can already be measured at the 1mg/mL concentration.
- 8 mg/mL or more: no difference between HA-NCs and HA-PH-NCs could be observed.

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• 8 mg/mL or more: no difference between HA-NCs and HA-PH-NCs could be observed.
Stability of the Nanocapsule Shell

NaCl 0.9%, Ringers Solution, DPBS Buffer, Human Serum and CASO Boullion

Experimental set up:
- 500 µL NCs dispersion (10^{13} NC per mL)
- 500 µl of the various media
- Incubation at 37°C for 24 h
- Centrifugation at 4000 rpm for 20 min
- Fluorescence signal in the supernatant

- fluorescence signal measured in the supernatant is almost the same as in the control samples (untreated samples)
- indicating the high stability of all polymeric nanocapsules
Bacteria Studies

MIC (µg mL⁻¹) of nanocapsules against
S. aureus ATCC 29213
S. aureus ATCC 43300
E. coli ATCC 25922

<table>
<thead>
<tr>
<th>Samples</th>
<th>S. aureus ATCC 29213</th>
<th>S. aureus ATCC 43300</th>
<th>E. coli ATCC 25922</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-NCs</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>HA-PH-NCs</td>
<td>62.5</td>
<td>62.5</td>
<td>250.0</td>
</tr>
<tr>
<td>PH-NCs</td>
<td>62.5</td>
<td>62.5</td>
<td>125.0</td>
</tr>
<tr>
<td>HES-NCs</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>HES-PH-NCs</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

PH-NCs and HA-PH-NCs:
- exhibits the same antibacterial activity against both strains of S. aureus → preventing growth at 62.5 µg ml⁻¹ in vitro
- shows a greater ability to inhibit the growth of S. aureus compared to E. coli
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**Work Flow - Nanoparticles**

Solvent evaporation method combined with the direct miniemulsion technique

- **Aqueous stabilizing molecule solution**
- **Ultrasonication**
- **Solvent evaporation**

Dispersed phase containing:
- Chloroform
- PLLA
- Octenidine

Octenidine

$\text{poly(L-lactide), PLLA}$
NPs - Characteristics

(A) Sodium dodecyl sulfate (SDS)
(B) Cetyltrimethylammonium-chlorid (CTMA-Cl)
(C) Polyvinylalcohol (PVA)
(D) Hydroxyethyl starch (HES)
(E) Human Serum albumin (HSA)
# Physico-chemical properties of PLLA-NPs

<table>
<thead>
<tr>
<th>PLLA-samples</th>
<th>Diameter, nm / Size distribution, %</th>
<th>Zeta potential, mV</th>
<th>Carboxylic groups per nm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLLA-PVA</td>
<td>280 / 21</td>
<td>-3</td>
<td>1.25</td>
</tr>
<tr>
<td>PLLA-SDS</td>
<td>160 / 26</td>
<td>-40</td>
<td>0.96</td>
</tr>
<tr>
<td>PLLA-CTMA-Cl</td>
<td>150 / 24</td>
<td>+42</td>
<td>1.18</td>
</tr>
<tr>
<td>PLLA-SDS</td>
<td>125 / 28</td>
<td>-36</td>
<td>0.86</td>
</tr>
<tr>
<td>PLLA-CTMA-Cl</td>
<td>115 / 25</td>
<td>+28</td>
<td>1.05</td>
</tr>
<tr>
<td>PLLA-PVA</td>
<td>230 / 16</td>
<td>+2</td>
<td>1.03</td>
</tr>
<tr>
<td>PLLA-HES</td>
<td>240 / 27</td>
<td>+4</td>
<td>0.79</td>
</tr>
<tr>
<td>PLLA-HSA</td>
<td>240 / 24</td>
<td>-3</td>
<td>0.99</td>
</tr>
</tbody>
</table>

- PLLA-NPs size between 125 and 240 nm depending on the used stabilizing molecules
- Zeta potentials around zero when using non-ionic stabilizing molecules
- Detection of carboxylic groups using a particle charge detector → COOH groups can further be used for coupling experiments
Release of Octenidine – HPLC Studies

1. Single measurements of all used ingredients
2. Measuring of the dispersion
3. Measuring of the supernatant

Comparison of:
- total amount of octenidine inside the NPs with the amount of octenidine found in the supernatant

- No octenidine found in the supernatant when using SDS or CTMA-Cl as stabilizing molecule → deletion of enzyme
- Increase in the amount of octenidine found in the supernatant by around 40% when using 10 mg per mL
- No difference between day 25 and day 40

500 μL NP (10¹³ NP per mL)
500 μl enzyme (1 or 10 mg/mL)
Gently shaken at 37 °C
Bacteria Studies

MIC (µg mL\(^{-1}\)) of nanoparticles against 
**S. aureus ATCC 29213**  
**S. aureus ATCC 43300**  
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</tr>
</thead>
<tbody>
<tr>
<td>PLLA-PVA</td>
<td>12.5</td>
<td>25.0</td>
<td>100</td>
</tr>
<tr>
<td>PLLA-HES</td>
<td>25.0</td>
<td>25.0</td>
<td>50.0</td>
</tr>
<tr>
<td>PLLA-HSA</td>
<td>12.5</td>
<td>50.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>

- Regardless of surfactant used, all particles showed antibacterial activity against both gram-positive and gram-negative bacteria
- Greater ability to inhibit the growth of *S. aureus* compared to *E. Coli*
Summary

• Synthesis of hyaluronic acid-based nanocapsules, which contain a fluorescent dye and a hydrophilic antimicrobial agent (polyhexanide)
• Synthesis of PLLA-based nanoparticles, which contain a hydrophobic antimicrobial agent (octenidine)
• Optimization in terms of:
  • nanocapsule/nanoparticles size
  • shell density
  • shell composition
  • encapsulation yield
• Successful release of polyhexanide from the HA-NCs in the presence of hyaluronidase studied by fluorescence spectroscopy and UV
• Successful release of octenidin from the PLLA-NPs in the presence of proteinase K detected by HPLC
• High stability of all polymeric NCs in various media
• all NCs and NPs showed antibacterial activity against both gram-positive and gram-negative bacteria
• Ongoing work: immobilization of NCs or NPs
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• All collaborators

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