MULTIBIOFUNCTIONAL BIODEGRADABLE NANOCONTAINERS FOR CONTROLLED HYDROPHOBIC DRUG DELIVERY

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Abstract

The investigation and application of new nano-technological products in medicine is very promising area which can seriously advance medical practice. The great amount of studies are devoted to obtaining of biodegradable “nanocontainers” capable for encapsulation and prolonged release of drug in the organism. Of significant importance is active targeting of such containers which could be achieved via covalent attachment of special biomolecules to their surface.

In the present study we have obtained nanoparticles based on different biodegradable polyesters. The possibility of hydrophobic substances and magnetic nanoparticles entrapment into obtained nanocontainers was proved. The particles degradation and release kinetics was investigated. In order to obtain nanoparticles with multibiofunctional surface different approaches were tested.

Keywords: biodegradable nanoparticles, drug delivery, biofunctional

1. INTRODUCTION

Formation and application of polymeric biodegradable nanoparticles for delivery and controlled release of drugs represent the important object of current research [1-3]. One of the inherent features of such particles is that they could bear in their inner space some sensitive or hydrophobic drugs to the in vivo targets, hiding these substances from aggressive media the human organism is [4-8]. It is known that some hydrophobic drugs are very promising, but their application is impossible without special delivery systems [6-8]. Thus the creation and investigation of such systems is of both scientific and practical importance.

Also it is important to supply such nanocontainers with special surface-associated biomolecules [9, 10], which are capable for highly specific interaction with special cell membrane receptors leading to accumulation of containers in the target tissue or organ. In current literature the covalent attachment of only one biomolecule type is usually performed [8-11]. From our point of view this is caused by insufficient amount of surface reactive groups. Nevertheless it is promising to construct a nanoparticle with multibiofunctional surface. It means that such surface should bear different specific and non-specific biomolecules in order to mimic cell-cell interactions and increase both specificity and efficiency of drug delivery.

In this paper we describe the formation of biodegradable nanocontainers capable for entrapment of hydrophobic drugs. The encapsulation efficacy and drug release as well as degradation kinetics were evaluated. Moreover the methods to obtain multibiofunctional particles are proposed and tested.

2. EXPERIMENTAL PART

2.1 Materials and instruments

All reagents (monomers, initiators, enzymes and biomolecules) as well as other substances used in this research were purchased from Fluka (Switzerland) and Sigma (Germany). Toluene, chloroform and methanol used for polymer synthesis were products of ZAO “Vecton” (Russia) and were dried over calcium hydride and distilled before application. THF used as eluent for GPC analysis was of HPLC grade and was...
purchased from Merck (Germany). For dialysis, particles purification and separation of non-reacted ligands from polymer conjugates the spin-columns (VIVASCIENCE, Sartorius Group, Germany) were utilized.

The $^1$H NMR spectra were recorded with Brucker AVANCE-400 instrument. For UV–vis measurements the UV mini-1240 spectrophotometer (Shimadzu, Japan) and was used. The Shimadzu liquid chromatograph consisted of LC-10AD pump, RID-10A refractometric detector and Waters Styragel HMW 6E analytical column was applied for GPC analysis. The T-18 basic homogenizer (IKA-Werk GmbH, Germany) and ultrasonic bath UZV-0.063/37 (37kHz, Russia) were used for emulsification. The dynamic light-scattering Malvern ZetasizerNano ZS (UK) apparatus was utilized for particles size evaluation.

2.2 Methods

2.2.1 The synthesis of polymers. The synthesis of poly(lactic acid) (PLA) as well as of copolymer of lactic and glycolic acids (PLGA) was performed via stannous octoate initiated ring-opening polymerization (ROP) in bulk of corresponding cyclic monomers. The reaction was conducted in vacuum-processed Schlenk tubes at temperatures above 120°C. The yields of polymer products precipitated in cold methanol are listed in Table 1. The PLGA composition was evaluated from $^{13}$C NMR DEPT135 spectra.

The synthesis of poly(caprolactone) (PCL) and poly(pentadecalactone) (PPDL) was performed via enzyme-mediated ROP of corresponding monomers as described earlier [12]. The Candida Antarctica lipase B (CALB) was used for catalysis of lactones polymerization.

The copolymerization of 2-deoxy-N-methacrylamidoglucone (MAG) with O-laurylmethacrylate (LMA) was performed via free-radical reaction similar to the previously published procedures [13]. The polymer products yields were 95-97 %. The MW was estimated to be 20 kDa.

2.2.2 Fabrication of nanoparticles via single emulsion technique. 100 mg of corresponding polymer was dissolved in 4 ml of chloroform and dispersed into 120 ml of water phase via simultaneous action of mechanical dispenser and ice filled ultrasonic bath for 30 min. The water phase consisted of 20 ml of ethyleneglycol and 100 ml of water in which sodium dodecyl sulphate (SDS, 0.5 wt%), and polyvinyl alcohol (PVA, 1 wt%) were dissolved. Then the chloroform was evaporated in rotary evaporator during 2 h to form the nanoparticles suspension.

2.2.3. Entrapment efficacy and release evaluation. Firstly the $Q_{\text{particles}}$ that is the quantity of drug entrapped into 1 mg of particles was evaluated via spectrophotometric detection. Then the amount of substance entrapped into the sample was calculated as $Q_{\text{entrapped}} = Q_{\text{particles}} \cdot m_{\text{particles}}$. The entrapment efficacy was obtained according to the following equation: EE (%) = $Q_{\text{entrapped}} \cdot 100 / Q_0$. The $Q_0$ is theoretical quantity of entrapmed drug: $Q_0 = C_0 \cdot V_0 / m_{\text{particles}}$, where $C_0$ is initial concentration of the drug solution and $V_0$ is volume of this solution taken for entrapment. The release (%) was evaluated as total quantity of drug detected in supernatant referred to the amount of entrapped drug. The release was conducted in 0.01 M phosphate buffer saline (PBS), containing 0.1% of SDS.

2.2.4. Modification of nanoparticles with biomolecules. Particles surface activation: 1. partial surface hydrolysis via keeping nanoparticles sample in 0.01 M NaOH for 0.5 h and further treatment with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC); 2. oxidation of surface-located glucose rings with NaJO4. In the case of PVA-coated nanoparticles modification the BSA carboxylic groups were activated with EDC. All biomolecules coupling reactions proceeded in 0.01 M borate buffer pH 10.0. The amount of bound protein or aminoacid was evaluated via Lowry test or reaction with ninhydrin, correspondingly.

3. RESULTS AND DISCUSSION

3.1 Polymers synthesis

PLA and PLGA were synthesized via ROP in the presence of stannous octoate (Table 1), while more hydrophobic PCL and PPDL were obtained by enzyme-mediated polymerization (Table 2). From our experience it is known that for nanoparticles preparation the polymer should not have MW more than 50 000,
but also should not be an oligomer. One can observe that obtained polymers satisfy these demands. It is also might be seen that lactide copolymerization with glycolide results in reiteration of monomer composition in the polymer product, the glycolide being slightly more reactive. Samples 1, 2, 4 and 5 were chosen for further particles preparation.

Table 1 Polymerization of L-lactide (LD) and its copolymerization with glycolide (GD) in bulk. The reaction time was 5 – 6 h for all samples, [M]/[SnOct₂] = 1000 mol/mol.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[LD]:[GD], mol.%</th>
<th>T, ºC</th>
<th>Yield, %</th>
<th>[LA]:[GA], mol.%</th>
<th>[n]</th>
<th>Mw×10⁻³ (GPC)</th>
<th>Mw/Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>120</td>
<td>61</td>
<td>100</td>
<td>0.19</td>
<td>13.0</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>80:20</td>
<td>120</td>
<td>92</td>
<td>70:30</td>
<td>0.13</td>
<td>6.1</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>95.5</td>
<td>140</td>
<td>94</td>
<td>90:10</td>
<td>0.10</td>
<td>5.4</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 2 Enzyme-mediated ring-opening polymerization of caprolactone and pentadecalactone in toluene solution. [Monomer] = 86 wt.%; [Monomer]/[Enzyme] = 10 wt./wt., T = 95 ºC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Monomer</th>
<th>t, h</th>
<th>Yield, %</th>
<th>[n], dl/g</th>
<th>Mw×10⁻³ (GPC)</th>
<th>Mw/Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>PPDL</td>
<td>2.0</td>
<td>88</td>
<td>0.9</td>
<td>35.0</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>PCL</td>
<td>5.5</td>
<td>92</td>
<td>0.4</td>
<td>25.4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

3.2 Nanocontainers fabrication. Encapsulation efficacy, release and degradation study

The nanoparticles were obtained via single emulsion evaporation method (Fig. 1, A). In order to obtain fine nanoparticles it is important to work at the minimum of surface tension. Thus the utilized SDS/PVA stabilizing system was investigated via du Noüy ring method (Fig. 1, B). It was found that concentrations of about 2-3 mg/ml are above the critical micelle concentration for this system and could be utilized for nanoparticles formation.

![Fig. 1](image1.png)

The typical size distribution diagrams of obtained particles are presented on Fig. 2. One can see that particles distribution (PD) is quite narrow. Moreover the PD and particles size are similar for both chemically and enzymatically synthesized polymers.

![Fig. 2](image2.png)
The batch of particles was obtained as based on the above described polyesters (Table 3). One can see that all particles have diameter less than 1 μm. There is also a good compliance between particle sizes detected by DLS and SEM methods.

Furthermore the entrapment efficacy and release were evaluated. For that the model hydrophobic drug – risperidone – was added to the polymer solution in chloroform prior to emulsification (Fig. 2, A). This substance possesses antipsychotic properties and is practically insoluble in water. Its entrapment into polymeric particles with subsequent controlled release is also of practical interest.

### Table 3 The characteristics of nanoparticles size, model drug (risperidone) entrapment and release (after 3 month) capability, obtained from different polyesters.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mean particles diameter (DLS), nm</th>
<th>PDI</th>
<th>Mean particles diameter (SEM), nm</th>
<th>Q&lt;sub&gt;articles&lt;/sub&gt;, mg/g of particles</th>
<th>EE, %</th>
<th>Release, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA</td>
<td>242</td>
<td>0.29</td>
<td>275</td>
<td>29</td>
<td>61</td>
<td>88</td>
</tr>
<tr>
<td>PLGA</td>
<td>520</td>
<td>0.18</td>
<td>543</td>
<td>35</td>
<td>63</td>
<td>98</td>
</tr>
<tr>
<td>PCL</td>
<td>410</td>
<td>0.22</td>
<td>472</td>
<td>61</td>
<td>81</td>
<td>90</td>
</tr>
<tr>
<td>PPDL</td>
<td>435</td>
<td>0.21</td>
<td>532</td>
<td>93</td>
<td>95</td>
<td>83</td>
</tr>
</tbody>
</table>

From the data in Table 3 it is seen that the chosen model drug could be successfully entrapped into the all types of obtained polymer particles. Nevertheless it appears that its entrapment into more hydrophobic PCL and PPDL is more efficient. At the same time the release of risperidone from particles based on PPDL seems to be less intensive. It is also obvious that drug release is more rapid from PLGA, which is known to be more amorphous, than from PLA, which has more crystalline nature.

It was also shown by us that hydrophobized by CTAB maghemite nanoparticles (6 nm, provided by Dr. Olga Osmolowskaya from St. Petersburg state university) could be entrapped into polymeric particles with efficacy about 80 %.

The simultaneous study of drug release and particles degradation kinetic profiles (Fig. 3) demonstrated that this processes are associated with each other. Thus by controlling the structure of the polymer we can govern the degradation kinetics and release of drug.

**Fig. 3** The kinetic curves of lactic acid formation (particles degradation), drug release and PLGA molecular weight (evaluated by GPC) decrease. Conditions: PBS pH 7.2, SDS 0.1%.

### 3.3 Nanocontainers biofunctionalization

On the next stage of this study the research was devoted to the attachment of model biomolecules (bovine serum albumine and glycine) to the particles surface. From our point of view, there are two ways to do this. First is the partial hydrolysis of the particles surface with subsequent activation of formed carboxylic groups with carbodiimide in order to activate them towards aminogroups of biomolecules (Fig. 5, A). The second approach is modification of reactive groups of macromolecular stabilizers that should be located on the surface of particles (Fig. 5, B). For example PVA, which is commonly utilized as such stabilizer, could be modified via reaction of its OH-groups with activated carboxylic groups of protein.

It was also of interest to utilize macromolecular stabilizer which have amphiphilic nature and gives the possibility to introduce quite reactive functional groups. Thus the new macromolecular stabilizer, namely pMAG-LMA copolymer, was synthesized. The LMA content was evaluated via integration of the signal corresponding to its methyl group and consisted 11 mol%. The PPDL-based particles obtained with utilization of pMAG-LMA as stabilizer showed similar particles size and PD to ones obtained with application of PVA (Fig. 4). This fact demonstrates that pMAG-LMA really serves as stabilizer and that it possibly could
be used for particles biofunctionalization. In order to activate surface located pMAG-LMA the particles were treated with sodium peridate to oxidize α-glycol part of glucose. The resulting aldehyde groups are quite reactive towards amino groups of biomolecules.

**Fig. 4** Size distribution diagram of particles obtained from PPDL

![Size distribution diagram](image)

**Fig. 5** Particles biofunctionalization: A – direct surface hydrolisis and activation; B – modification of surface-located macromolecular stabilizer.

![Particles biofunctionalization](image)

The results of abovementioned nanoparticles biofunctionalization approaches comparison are presented in Table 4. One can observe that while the particles sizes are quite similar, the quantity of biomolecules coupled are notably differ from each other. The most effective protein and aminoacid coupling was detected in the case of surface-located oxidized pMAG-LMA modification, while in the case of PVA modification only the minimal amount of bound protein is detected. The latter could be explained by relatively low reactivity of hydroxyl groups in comparison with other chemistries. The direct surface activation by hydrolysis gives the middle result. It seems that in this case the amount of protein coupled is limited by steric hindrance. At the same time the coupling to hydrophilic oxidized pMAG „tails“ which are exposed to the water phase gives more steric freedom and thus greater capacity. This assumption is certified by the close capacities of the discussed particles towards glycine.

In order to compare the capacities of B1 and B2 samples regarding to the BSA molecules chemisorption the corresponding isotherms were evaluated and plotted (Fig. 6). From the obtained curves it is obvious that oxidized hydrophilic surface of B3 sample gives possibility to attach greater amount of protein then B1 sample. In the latter case the surface is saturated with protein, while in the former one it seems that some reactivity still remains.

**Table 4** The dependence of the coupled ligand quantity on the activation method. In all cases the biomolecules solution concentration was 2 mg/ml (solution volume 1.5 ml; 20 mg of particles).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Polymer</th>
<th>Activation method</th>
<th>Mean particles diameter (DLS), nm</th>
<th>( Q_{\text{coupled}} ) (BSA), ( \mu g/mg )</th>
<th>( Q_{\text{coupled}} ) (Gly), ( \mu g/mg )</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>PLA</td>
<td>hydrolysis + EDC</td>
<td>421</td>
<td>7.4</td>
<td>27.2</td>
</tr>
<tr>
<td>B2</td>
<td>PLGA</td>
<td>PVA + activated BSA</td>
<td>375</td>
<td>1.8</td>
<td>Not conducted</td>
</tr>
<tr>
<td>B3</td>
<td>PPDL</td>
<td>oxidized pMAG-LMA</td>
<td>410</td>
<td>13.2</td>
<td>34.7</td>
</tr>
</tbody>
</table>

The greater amount of glycine coupled in comparison with BSA is explained by the small size and, correspondingly, the simplified diffusion of the first one. Interestingly that immersion of the particles B3
modified with BSA to the glycine solution (1 mg/ml) was leading to the additional coupling of 11 μg of aminoacid per 1 mg of particles. This fact proves that by using this approach the covalent attachment of several ligands of different dimensions is possible.

CONCLUSION
Different types of polyesters were synthesized and used for nanoparticles fabrication via optimized single emulsion evaporation technique. The ability of such particles to entrapp and continiously release hydrophobic model drug was demonstrated. The correlation of degradation and drug release kinetics is shown. Finally, different approaches for particles biofunctionalization were tested. It was shown, that application of hydrophilic stabilizers on the particles preparation stage gives the possibility to couple several biomolecules at controllable ammounts.

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