Novel approaches to microencapsulation of animal cells using smart chitosan-poly(vinyl alcohol) copolymers and RGD-peptides

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INTRODUCTION

Microencapsulation is a promising technique for a number of biomedical applications, namely diabetes treatment, somatic gene therapy, tissue engineering, anticancer drug screening using microencapsulated multicellular tumor spheroids (Tsoy 2010). Advantages of microencapsulation as a 3-dimensional (3D) growth technique are chemically and spatially defined 3D network of extracellular matrix components, cell-to-cell and cell-to-matrix interactions which govern differentiation, proliferation and cell function *in vivo*. These, in fact, are lost under the simplified 2D conditions. Microencapsulation was widely used to support growth of various cells, such as insulin-producing pancreatic beta-cells, parathyroid cells, hepatocytes, stem and hybridoma cells.

However, cell microencapsulation technique still requires an improvement of procedures, materials and devices for microcapsule preparation. Chitosan-based polymers could be a good alternative to replace PLL or other rather expensive polypeptides, since natural polysaccharides (e.g. chitosans) are cheaper. Moreover, a direct comparison showed that the toxic effect of alginate-PLL membrane was significantly higher than those for chitosans. However, high molecular weight chitosans are soluble only at acidic pH value while cell encapsulation procedure should be carried out under physiological conditions (pH 6.8-7.2). The grafting of chitosans with biocompatible water-soluble synthetic polymers, e.g. poly(vinyl alcohol) (PVA), could be a good approach. Unlike chitosans, these copolymers are soluble at neutral pH.

Thus, the aim of the current study was to fabricate biocompatible smart microcapsules based on novel chitosan-graft-PVA (chitosan–g-PVA) copolymers and to show that these microcapsules can support animal cell growth at long-term cultivation *in vitro*.

MATERIALS AND METHODS

Sodium alginate (medium viscosity, 3,500 cps at 25°C), EDTA sodium salt, BSA, mouse IgG antibodies, horseradish peroxidase-linked goat anti-rat IgG conjugate were from Sigma-Aldrich, Germany. Calcuim chloride was from Panreac (Spain). PBS (pH 7.2), Tween-20, NaCl and Trypan Blue were from PanEco (Russia), Serva (Germany), REACHEM (Russia) and Flow Laboratories (Germany), respectively. Oligochitosan (M_w 3.5 kDa, DD 89%) and peptides containing RGD-motif were kindly provided by Prof. A. Bartkowiak (Poland) and Prof. S.Burov (St-Petersburg, Russia), respectively. For synthesis of chitosan-g-PVA copolymers, α-chitin (M_w 650 kDa) was purchased from COMBIO (Russia), and poly(vinyl acetate) (PVAc), M_w 350 kDa, was from PLASTPOLYMER (Russia). Sodium hydroxide was from Merck (Germany), PVA (Mowiol 66-100; M_w 100 kDa) was from Sigma-Aldrich (Germany).

Cell cultures. A series of cell lines were used in this study, namely Siberian mountain goat kidney cell line (PSGK-60); baby hamster kidney cells (BHK-21); rat-mouse hybridoma cells (1D2) producing monoclonal antibodies (MAbs) against IgG heavy chain; a mouse macrophage-like cell line (P388D1); mouse melanoma cells (M3); human breast adenocarcinoma cell line (MCF-7). Myeloma cells (Sp2/0), and two human T-lymphoblastic leukemia cell lines (CCRF-CEM and camptothecin resistant CEM/C1 subline) were also used. Cells were cultured in MEM or DMEM (Sigma-Aldrich, Germany) in CO_2- incubator at 37 °C. All media were supplemented with 10% fetal bovine serum (FBS) from HyClone (USA).

Synthesis of chitosan–g-PVA copolymers. Two graft copolymers of chitosan and PVA, i.e. chit-g-PVA_1 and chit-g-PVA_{20}, (PVA/chitosan molar ratio 0.92 and 20.5, respectively) were obtained by Solid-State Reactive Blending (SSRB) method (Zaytseva-Zotova 2012).

Cell microcapsulation and cultivation. Cell precipitate ($1\text{-}6\times10^6$ cells) was mixed with a sterilized NaAlg solution (2 wt.-%, 2 mL), and the mixture was added into a $CaCl_2$ solution (0.5 wt.-%) using a device with a coaxial air flow (2×10^5Pa) or electrostatic bead generator. CaAlg beads were incubated with chit-g-PVA copolymer or oligochitosan solution (25 mL, 0.1-1.2 wt.-%, 5–10 min), washed with 0.9 % NaCl solution, treated with EDTA (50 mM solution, 10 min), again washed and transferred to the culture medium. Cells were cultivated within microcapsules for 3-4 weeks. To measure cell density, microcapsule aliquots (0.1 mL slurry) were destroyed and cells counted. Microcapsule size distribution and membrane thickness were calculated using optical microscope (Reichert Microstar 1820 E, Germany). MAbs concentration produced by encapsulated 1D2 hybridoma cells in culture medium was examined by ELISA as described earlier (Zaytseva-Zotova 2012).

RESULTS AND DISCUSSION

To combine chitosan pH-sensitivity and PVA water solubility at physiological conditions (pH 7.2), PVA-

g-chitosan copolymers were synthesized. Water-soluble at room temperature fractions of the obtained products were characterized by FTIR, elemental analyses and gel penetration chromatography. FTIR spectroscopy showed that DD of both copolymers was up to 98%. Polymer-analogous transformations under these conditions were accompanied by a formation of an extensively grafted co-polymeric system. FTIR sample spectra contained characteristic absorption PVA and chitosan bands (Fig. A, B). Fig. 1A shows FTIR spectra for chit-g-PVA_1 copolymer (curve 1) and chitosan (curve 2). Fig.1B shows FTIR spectrum for chit-g-PVA_{20} sample (curve 2) compared to a model chitosan/PVA 20/80 (w/w) mixture (curve 1). Chitosan contents in the copolymers were found to be 80 and 15 wt.-% for chit-g-PVA_1 and chit-g-PVA_{20}, respectively. Calculations from results of elemental analysis using C/N ratio for pure chitosan and chit-g-PVA copolymers were in a good agreement with FTIR data. Copolymers M_w according to GPC were 124 kDa and 190 kDa for chit-g-PVA_1 and chit-g-PVA_{20}, respectively. Both copolymers were water-soluble at physiological conditions (pH 7.2, room temperature).

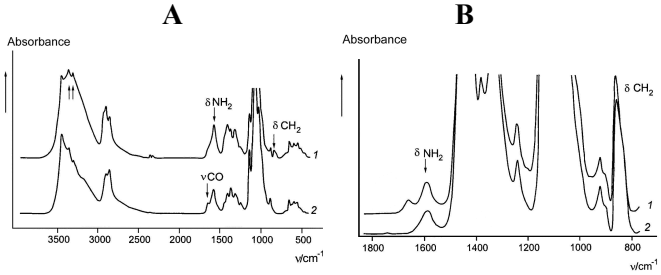

Fig. 1. FTIR spectra of chit-g-PVA_1 copolymer (curve 1) and chitosan (curve 2) (A); chitosan/PVA 20/80 (w/w) model mixture (curve 1) and chit-g-PVA_{20} (15 wt.-% Chit) copolymer (curve 2) (B).

Microcapsule membrane thickness depended on polycation concentration, its type and incubation time. Stable microcapsules with an optimal membrane thickness were obtained after CaAlg beads incubation in oligochitosan, chit-g-PVA_1 and chit-g-PVA_{20} solutions for 5, 5 and 10 min, respectively (Table 1).

Table 1. Membrane thickness of microcapsules in function of polycation type and incubation time.

Polycation sample	Microcapsule mean d [μm]	Polycation solution conc [%]	Membrane thickness as function of incubation time [μm]		
			5 [min]	7 [min]	10 [min]
Oligochit	900 ± 75	0.10	78 ± 5	112 ± 6	n.d. [a]
		0.20	89 ± 5	135 ± 6	n.d.
Chit-g-PVA_1 [b]	780 ± 50	0.20	27 ± 5	–	54 ± 5
		0.25	51 ± 5	76 ± 5	n.d.
		0.50	81 ± 5	–	n.d.
Chit-g-PVA_{20} [c]	1150 ± 80	0.75	–	37 ± 5	66 ± 5
		1.15	–	42 ± 5	87 ± 5

[a] Membrane was not detected [b] Chitosan/PVA ratio was 80/20 (w/w), M_w = 124 kDa [c] Chitosan/PVA ratio was 15/85 (w/w), M_w = 190 kDa.

Since chit-g-PVA copolymers were pH-sensitive due to chitosan, the microcapsules easily dissolved by slightly increasing pH value up to 8.0 – 8.2. To test microcapsules for their capability to support cell growth, various cells differed in their origin (human, mouse, etc.) and growth properties (suspension culture or monolayer) were chosen for microencapsulation. Optimal initial cell density for microencapsulation was (1.0 ± 0.2) × 10^6 cells mL^{-1} of the NaAlg solution. After cultivation of microencapsulated cells for 3-4 weeks multicellular spheroids for almost all selected cell lines, except M3 mouse melanoma cells, were generated. M3 cells grew on the inner microcapsule membrane but did not fill the microcapsule volume, since their growth was suppressed. To promote M3 spheroid formation, we induced cell aggregation by cell incubation with RGD-peptides (0.1-100 uM) for 48-72 h before microencapsulation. As a result, previously obtained cell aggregates with mean size 110 um were encapsulated to get MS in microcapsules. 8 different cell lines, including tumor cells (Fig.2), were cultured in the microcapsules for 3 weeks, and their growth kinetics was studied. Thus, cell concentration increased 10-fold in first 5 days, and reached 7 × 10^6 cells mL^{-1} and 4.5 × 10^6cells mL^{-1} for BHK-21 and 1D2 hybridoma cells, respectively, by day 11^{th}.

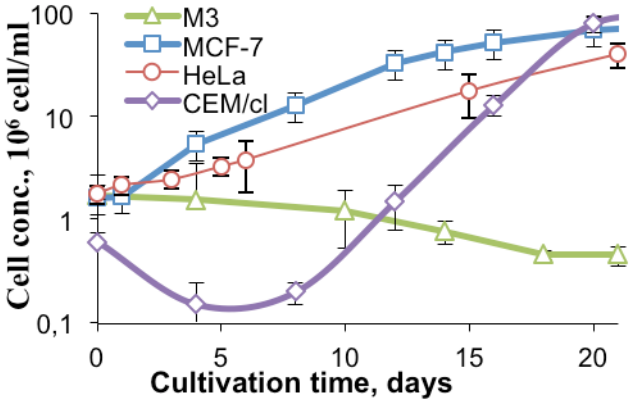

Fig. 2. Growth of tumor cell lines in microcapsules.

CONCLUSION

Thus, microcapsules based on chitosan-g-PVA copolymers were stable at long-term cell cultivation and provided multicellular spheroids formation which could be easily released by slightly rising pH of the culture medium up to pH 8.0 – 8.2.

REFERENCES

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- Zaytseva-Zotova et al. (2012) *Biocompatible Smart Microcapsules Based on Chitosan-Poly(Vinyl Alcohol) Copolymers for Cultivation of Animal Cells.* Advanced Engineering Materials 13 (12) B493-B503.

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