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The Deposition of Chlorhexidine on Chemically Modified Thermosensitive PolyNIPA Microgels Assessed by EDXS in Scanning Electron Microscopy

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***SUMMARY*. The deposition of chlorhexidine base on thermosensitive N-isopropylacrylamide polymeric microparticles was assessed in this study using energy dispersive X-ray spectrometry (EDXS) in scanning electron microscopy (SEM) setting. Three different types of polymer were synthesized. The PN1 was a polymer with terminal anionic groups resulting from potassium persulphate initiator. The PN2 was synthesized with 2,2'-azobis(2-methylpropionamidine)dihydrochloride, what resulted in cationic amidine terminal groups. The PN3 had anionic terminals, however increased hydrophobicity was maintained with N-tert-butylacrylate functional groups. The thermosensitivity of the polymer-chlorhexidine complexes was confirmed by the turbidimetric assay. The deposition patterns, observed in/on the polymers give the assumption to develop the applications of evaluated polymers as factors influencing chlorhexidine release from the topical formulations.**

INTRODUCTION

Chlorhexidine is widely used in dentistry, and in limited range in dermatological applications, as well as disinfectant in medical prophylactic procedures, due to its efficient activity against Gram-positive and Gram-negative bacteria, as well as against fungi and enveloped viruses [1-3]. High topical levels of chlorhexidine may be toxic, however in usually applied concentrations it is well tolerated by mucosa and the skin. However the local increase of chlorhexidine concentration in the mucosal area or on dysfunctional area of the skin, especially in long term therapy, may lead to unacceptable levels of chlorhexidine also in the central compartment. Due to this fact new forms of chlorhexidine are developed, with specific polymeric carriers - synthesized or formed from natural polymers. Reddy et al. proposed gelatin bioresorbable chips to enhance bone gain for periodontal regeneration [4]. Interesting form of carrier for prolonged release of chlorhexidine evaluated Meng *et al.* –the chlorhexidine acetate is intercalated in montmorillonite, a mineral applied as a filler in thermal dressings– the intercalated complex chlorhexidine-montmorillonite strongly inhibited growth of wide spectrum of pathogens [5]. Copolymers of hydroxyethyl methacrylate and methyl methacrylate were synthesized and applied to fabrication of a membrane-controlled, reservoir-type controlled-release delivery system to enable intraoral use of chlorhexidine [6]. New polymeric carriers were synthesized using urethane dimethacrylate-triethylene glycol dimethacrylate resin system [7]. Some authors evaluated special inclusion complexes based on porous silica and cyclodextrins [8]. Other porous systems were evaluated form methacrylate derivative polymer [9]. The research on chlorhexidine carriers for local delivery of this disinfectant to mucosa and skin is still an interesting field of study. In our previous work we evaluated systems with chlorhexidine based on methylcellulose and polyacrylic acid [10]. Some attempts were also made to perform preliminary assessment of chlorhexidine release from N-isopropylacrylamide beads, however the placement of chlorhexidine in the microgel still was not elucidated [11].

Microgels are identified as colloidal, stable cross-linked polymeric networks, which size is in the scale of nanometers to micrometers. Within the macromolecule the deswelling process is controlled by diffusion - the rate of the collapsing of the particle depends on the dimensions of pores in the polymeric matrix. The

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swelled microgels may contain up to 95 % of water, whereas in the deswollen state the water quantity decreases to *ca.* 20 % of the mass of polymeric matrix. Between numerous thermosensitive entities poly(N-isopropylacrylamide) (polyNIPA) is one of the most utilized macromolecules, characterized by reversible volume phase transition at specified temperature close to 32 ºC volume phase transition temperature (VPTT). The microgel thermosensitive particles may be applied in many medical devices, including drug forms for topical use. The collapsing and expanding of the macromolecule in the aqueous environment gives possibility to release various bioactive molecules in the controlled manner [12]. Synthesis of microgel particles, performed in the presence of different co-monomers, enables modification of polyNIPA characteristics; consequently anionic, cationic or hydrophobic polyNIPA polymers are obtained [13,14]. Newly synthesized colloidal microgels consisting of a crosslinked polymer networks have recently received attention as environmentally responsive systems, including different stimuli, *i.e.:* temperature, pH, enzymatic activity or concentration of specified chemical molecules. Due to bibliographic data the microgels have potential in drug release to the skin. They may be of particular importance where the skin barrier is compromised, as in disease state, or in wound management. The controlled delivery of actives to the skin can provide therapeutic levels where required and minimize systemic uptake [15,16]. The affinity of some bioactives to the microgels was modified by the temperature of the process of absorption [17], whereas the morphology of the polymeric material was modified by changing of the co-monomers, and reaction conditions [18]. Wide spectrum of literature covers release experiments and evaluations of various absorption isotherms to identify the proper composition of bioactive substance *e.g.* chlorhexidine implemented or incorporated into polymeric drug carrier [19-22]. Few communications considers the EDXS as a source of information on chlorhexidine deposition on polymeric material presumed for controlled drug delivery [23-25], including one of our paper [10]. Fay *et al.* evaluated microspheres loaded with chlorhexidine and composed of polyvinyl alcohol [23], other authors analyzed effect of chlorhexidine on nanoleakage of luting cements in dentistry by the mean of EDXS [24]. Interesting application of EDXS in the context of chlorhexidine level in material of high chemical diversity proposed Dynes et al. for quantitative mapping of chlorhexidine in natural river biofilms, however in described settings the transmission electron microscopy was used [25].

The aim of the present study was to investigate the deposition of weakly soluble base chlorhexidine crystals on the surface and inside of the matrix of polyNIPA polymers synthesized in the presence of acidic initiator or basic initiator, and compared to the deposition of chlorhexidine on the polyNIPA-co-tert-butylacrylate polymer, using EDXS device within SEM setting.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide 97 % (Aldrich), N-tert-butylacrylamide 99 % (Acros organics), N,N'-methylenebisacrylamide 99 % (Aldrich), potassium persulphate 98 % (BDH Laboratory Suppliers (GPR™), and 2,2'–azobis(2-methylpropionamidine) dihydrochloride 97 % (Aldrich) and chlorhexidine base (Aldrich) were obtained from commercial suppliers, and used without further purification. Dialysis bag for purification of microgels of molecular weight cut off, MWCO: 12000-14000 Da was obtained from Visking Medicell International Ltd. Deionized water obtained from the osmotic column TKD 9000 was applied in all the procedures.

Synthesis of Poly(NIPA) microgels

Poly(NIPA) microgels particles were synthesized by surfactant free dispersion polymerization (SFDP) in deionized water at 343 K, under an inert nitrogen atmosphere – the detailed procedure was described formerly [26]. The free radical initiator was placed in a 1 L, three-necked round-bottomed flask and stirred continuously at 120 rpm. Pre-dissolved NIPA, and eventually co-monomer tert-butylacrylamide, and N,N'-methylenebisacrylamide as a crosslinker, were dissolved in 200 mL deionized water under stirring and then added to the reaction vessel. After 6 h the dispersion was cooled to room temperature and filtered through glass wool. Further purification was done by dialysis against deionized water until the conductivity was less than 1 μS/cm. Dry weight analysis of the microgels revealed that the dispersion concentration is in order of 0.52 % (w/w). PN1 polymer was characterized as an polymer with terminal anionic functional groups. The PN2 was synthesized in the presence of 2,2'-azobis(2methylpropionamidine) dihydrochloride, which resulted in cationic amidine terminal functional groups, whereas the PN3 may be characterized as the polymer with anionic terminal functional groups with ad-

ditionally increased hydrophobicity according to functional tert-butyl acrylate groups implemented in the course of synthesis. For the evaluation of the completion of polymerization process the IR spectra assessments were performed. The presence of vinyl groups and other functional groups were analyzed and compared to the substrates of the reaction. The spectra of pure chlorhexidine, PN1, PN2, and PN3 were further compared to the freeze-dried complexes: PN1-CHX, PN2-CHX, PN3-CHX. The obtained polymers in dispersions were applied as they were, to be loaded by the chlorhexidine base, for 72 h. The composition of obtained mixtures is presented on the Table 1.

The mixtures were consequently freeze-dried for 24 h, using MINI LYOTRAP LF/LYO/02/1 with vacuum pump model RV5, in high vacuum mode, at 50 % power setting, with vacuum values in the range of 1×10^{-1} to 1×10^{0} mbar (*i.e.* 1×10^{1} to 1×10^{2} Pa). The VPTT for obtained mixtures was assessed by turbidimetric analysis of the diluted microgel dispersions without and with the addition of chlorhexidine carried out in the HACH 2100 Turbidimeter, stepwise, with 1 °C increase in every 15 min.

SEM and EDXS evaluation

Surface and morphology of freeze dried samples was assessed by the SEM. The electron microscopes FEI QUANTA 200 3D for uncovered samples and FEI SIRION for samples covered by gold particles were used. Energy Dispersive X-Ray Spectrometry (EDXS), connected to SEM device, was applied for evaluation of the presence of chlorine element in the loaded microgels. The X-rays emitted from the samples under the electron bombardment were collected with a liquid nitrogen-cooled state detector in the SEM equipment - FEI SIRION XL SFG, with INCA EDXS analyzer, X-Sigma Oxford Instruments, and analyzed according to the assessed energy. The number of X-rays detected per channel (10 electron volts/channel) versus assessed energy expressed in KeV were arranged in histograms, and interpreted to evaluate the presence of chlorine element [27].

RESULTS AND DISCUSSION

During the SFDP the unsaturated compounds would be saturated and the bands higher than 3000 cm-1 should not be observed, if there are not any other strong absorbancies. The peaks observed in the monomer at 3104.62 cm^{-1} and 3030.65 cm^{-1}, assigned to the stretching vibrations of unsaturated C=C bond (reference 3080 cm^{-1} and 3020 cm^{-1}) vanished from the spectrum after reaction. Also the band of 1620.88 cm^{-1}, assigned as the absorption of stretching vibrations of the C=C bond, and peak at 1409.84 cm^{-1} of the in-plane deformation vibrations of the C-H bond disappeared from the spectrum of the product, comparing to the monomer. Also the peak near 1300 cm^{-1} accompanying the peak 1409.84 cm^{-1} vanished from spectrum after saturation. Both out-of-plane deformation vibration of the C-H bond at the 986.91 cm^{-1} and 918.46 cm^{-1}, as well, were not observed in the polymer specter after finishing the reaction. Also the frequency of carbonyl group accompanied by the vinyl, recognized at the frequency around 960 cm^{-1} disappeared, as well as the frequencies 847.56 cm^{-1}, 808.53 cm^{-1}, and 664.19 cm^{-1}. This was evaluated for the PN1, PN2 and PN3, to confirm the course of the reaction. The PN1, PN2, PN3 polymers were loaded with chlorhexidine, and freeze-dried. The obtained PN1-CHX, PN2-CHX, and PN3-CHX preparations were analyzed with the IR spectrophotometry. In Table 2 most relevant observations are presented. The imine stretching vibration of the group C=N-H usually occurs in the region 3400-3300 cm^{-1} [28]. Detailed evaluation of infrared assessments and results was presented in our former work [26].

The respective absorbance of N-H stretching in chlorhexidine molecule at 3468 cm^{-1}, shifted a bit according to the protonation, was moved to the lower values on the specter of PN1-CHX, and was covered by another signals; this may confirm some associations between the NH groups of chlorhexidine and functional groups of the polymer. Additionally in the region 1690-1640 cm^{-1} there is a shift observed between chlorhexidine and PN1-CHX; the chlorhexidine

Sample	PN1 (mg)	PN2 (mg)	PN3 (mg)	CHX (mg)	Water (mg)
CHX-PN1	100.0	-	-	50.0	2000.0
CHX-PN2	-	100.0	-	50.0	2000.0
CHX-PN3	-	-	100.0	50.0	2000.0

Table 1. The composition of evaluated preparations of chlorhexidine with synthesized polymers.

Assignation group	Reference range	CHX	PN1-CHX	PN2-CHX	PN3-CHX
N-H str. vibr. of C=N-H	3400-3300	3468	-	-	-
C=N str. vibr. of C=N-H	1690-1640	1664	1636	1635	1631
Guanidines str. vibr. =N-C=N-	1685-1580	1598	1534	1532	1535

Table 2. The changes in the IR spectrum after loading process of the polymers, with chlorhexidine.

band of C=N stretching vibration at 1664 cm^{-1} in the complex is observed at 1636 cm^{-1}, what is an important prerequisite to claim the ionic character of the interaction [29-31]. Also the stretching vibration characteristic for the guanidines =N–C=N– shifted from 1598 cm^{-1} in chlorhexidine IR spectrumr to 1534 cm^{-1} in chlorhexidine-PN1 IR specter [31]. Similar relations were observed for the PN2-CHX and PN3-CHX complexes. This information confirms the ionic nature of the interactions between the assessed polymers and chlorhexidine; this range of pNIPA infrared specter was evaluated by other authors to elucidate the conformation changes in the molecule, when collapsing during phase transition, induced by the temperature factors [32].

The obtained values of VPTT were in the range between 31 °C and 42 °C for the PN1 and PN2, whereas the VPTT of PN3 was wide, and the collapsing of the microparticles was observed up to the temperature of *ca.* 36 °C, where the turbidity did not increase anymore, indicating the stable minimal hydrodynamic diameter of the particles of PN3 over the temperature of 36 °C.

During the synthesis performed by the SFDP method, and consequent loading of the product with chlorhexidine, preparations presenting different micromorphology were obtained. In the morphological study by SEM, the synthesized PN1 was identified as continuous structure with some droplets of microspheres, coming from the SFDP process, however in general, the structures would be assessed as continuous hydrogel with some increments, without specific spherical structures (Fig. 1, PN1).

Oppositely, in the case of PN2 and PN3 the material was not continuous, consisting of numerous spheres in dimensions between 0.25 μm to 2.5 μm, with some of higher diameter. Detailed morphology of this kind of material was evaluated in former paper [10]. The PN1 material was rather planar, of thickness in the range 1-10 μm and compact, with some empty areas of diameter in the range between 10 and 100 μm. With the increase of the optical magnification the small spherical structures were revealed. The loaded freeze-dried polymer PN1-CHX is

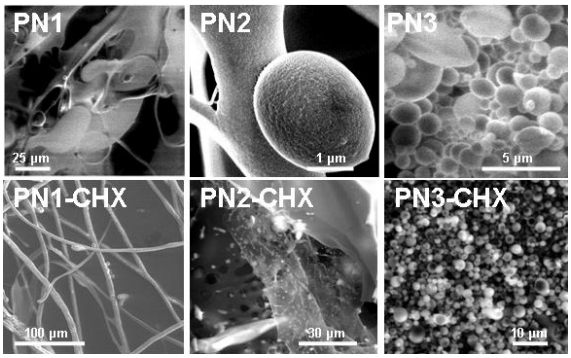

Figure 1. The obtained microgels before loading with the chlorhexidine: PN1, PN2, PN3 (first row), and microgels loaded with the chlorhexidine: PN1-CHX, PN2-CHX, PN3-CHX (second row).

presented in the Figure 1 in the second row. After loading the polymer PN1 with chlorhexidine and freeze-drying, the resulting material was elongated in fibers-like structures, of diameter in the range 2.5-5.0 μm and the length over 500 μm. In the case of PN2 also the planar structures were observed - similarly as in the case of PN1. The magnification revealed spherical structures embedded into a hydrogel matrix. The polymeric material loaded with chlorhexidine PN2-CHX was as well planar in general view, but in SEM assessments small spheres was revealed, with subtle crystals present in the neighborhood, which were assigned as chlorhexidine crystals in EDXS assessments; the crystals were also embedded into the polymeric matrix (Fig. 1, second row, PN2-CHX). The PN3 material was most interesting in both cases, *i.e.* before (Fig. 1, first row, PN3) and after loading (Fig. 1, second row, PN3-CHX), and some crystals were identified inside of the particles. The PN3 consisted of spheres of mean diameter 1.67 μm, 76.9 % of the particles was in the range between 1.0 μm and 2.0 μm. The diameter of the non-polymeric material included into the polymeric matrix was in the range below 1 μm. In this case we suggest that during the loading of the microspheres by the chlorhexidine, the chlorhexidine was partially dissolved, whereas after the freeze-drying it was entrapped inside of the polymeric spherical net in the way similar, as it is in the case of the so called intercalation components

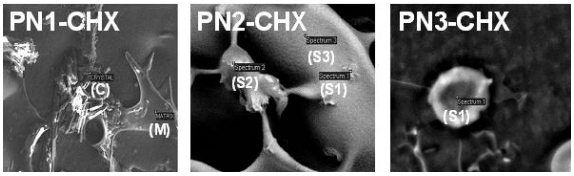

Figure 2. The deposition of chlorhexidine on the obtained polymers: PN1 (image PN1-CHX), PN2 (image PN2-CHX) and PN3 (image PN3-CHX) - the areas, where EDXS assessments were performed are marked: C, M, S1-S3, according to the Table 3.

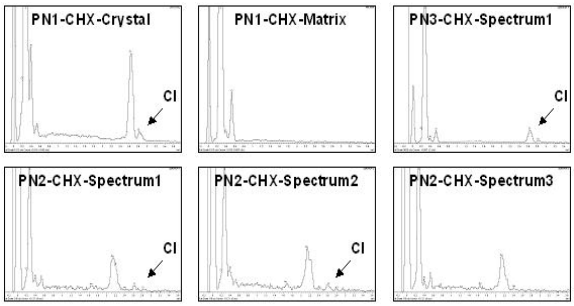

Figure 3. The EDXS assessments in various regions of obtained and loaded microgels PN1, PN2, and PN3. The respective details are given in the Table 3.

(Fig. 1, second row, PN3-CHX). This phenomenon could be applied to control the release of chlorhexidine from the formulation. As it was mentioned before, the chlorhexidine was presumably embedded into the polymeric matrix, both in the case of microparticles, and planar hydrogel structures.

Using the EDXS assessments, the deposition of the chlorhexidine particles was confirmed. However the chlorhexidine was deposited rather on the surface of PN1 and PN2, not inside of the polymeric matrix. In the case of PN3, the chlorhexidine was embedded into the polymeric structure of the microparticles. In the measurements, the presence of chlorhexidine was definitively confirmed. As it can be evaluated from the image on the Figure 2 (PN1-CHX), all the needle-like shaped, narrow crystals of chlorhexidine were observed in aggregates on the surface of the polymeric material. The chlorhexidine was distributed on the surface of the material.

The EDXS measurements revealed that chlorhexidine was rather on the surface of the polymeric matrix, whereas the actual polymeric material was not loaded by the chlorhexidine: compare the regions Crystal (C) and Matrix (M) and the respective results of chlorine element assessments in this regions: PN1-CHX-Crystal and PN1-CHX-Matrix. We assume that the PN2 polymer absorbed the CHX both in the form of visible crystal (Fig. 2, PN2-CHX), and in the form of subtle dispersion - all regions assessed by the EDXS presented significant level of chlorine element comparing with the baseline - see PN1-CHX-Spectrum 1 to 3 on the Fig. 3, and respective microimages (Fig. 2). The PN3, in magnification, presented some increments in circular microparticle visualized on the Fig. 2, section PN3-CHX. According to the EDXS measurements, chlorine element was identified within the particle: PN3-CHX-Spectrum 1 (Table 3, Fig. 3). The obtained physical inclusions of chlorhexidine in the polymeric matrix have the potential for the application in topically applied devices for controlled drug release, and will be further examined in the terms of drug release. Respective spectra from EDXS device are attached in the Figure 3.

The evaluation of deposition of bioactive, *i.e.* chlorhexidine base, on or in the polymeric matrix is possible by the mean of EDXS. This method gives insight into placement of chlorhexidine microcrystals, when loaded to the polymeric matrix. Due to the simultaneous observation of microscopic SEM images and EDXS assessments the inclusion of chlorhexidine was identified in the case of three various polymers.

Preparation	Assessed region*	Elements			
		C	N	O	Cl
PN1-CHX	Crystal (C)	58.17	32.26	0.00	9.57
	Matrix (M)	80.44	00.00	19.56	0.00
PN2-CHX	Spectrum 1 (S1)	61.55	17.39	6.80	14.26
	Spectrum 2 (S2)	62.56	20.33	2.90	14.21
	Spectrum 3 (S3)	71.12	16.35	12.53	0.00
PN3-CHX	Spectrum 1 (S1)	76.47	6.85	13.21	3.47

Table 3. The assessed amounts of elements in some regions of the polymeric matrix, all results in weight %, (*visualized on the Figure 2).

The highest levels of chlorine element, which reflected the presence of chlorhexidine in the sample, was determined in polymer PN2 characterized by cationic amidine terminal groups – interestingly, due to EDXS observations, the chlorhexidine was present both in the form of crystal aggregates and dispersed in the polymeric matrix. In the case of highly hydrophobic PN3 polymer, the specific aggregates with chlorhexidine closed within the polymeric microsphere were observed. The EDXS assessments may be applied for evaluation of patterns of chlorhexidine absorption to newly synthesized thermosensitive polymers.

CONCLUSIONS

Deposition of chlorhexidine base on/in the polymeric matrix may be performed by the mean of EDXS. The placement of chlorhexidine microcrystals, when loaded to the polymeric matrix may be assessed this way. Simultaneous observation of microscopic SEM images and EDXS data gives information on the patterns of deposition of chlorhexidine on the polymeric particle. The highest levels of chlorhexidine were determined in polymer PN2 with cationic amidine terminal groups. The highly hydrophobic PN3 polymer, implemented the specific aggregates with chlorhexidine closed within the polymeric microsphere. The EDXS assessments may be applied for evaluation of patterns of chlorhexidine absorption on newly synthesized N-isopropylacrylamide derivatives.

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