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Trimethyl and carboxymethyl chitosan carriers for bio-active polymer–inorganic nanocomposites

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ABSTRACT

The carrier properties of carboxymethyl chitosan (CMC) and trimethyl chitosan (TMC) in combination with polyoxometalates (POMs) as inorganic drug prototypes are compared with respect to the influence of polymer matrix charge and structure on the emerging composites. A direct crosslinking approach with TMC and $K_6H_2[CoW_{11}TiO_{40}]\cdot 13H_2O$ ($\{CoW_{11}TiO_{40}\}$) as a representative anticancer POM affords nanocomposites with a size range of 50–90 nm. The obtained POM–chitosan composites are characterized with a wide range of analytical methods, and POM encapsulation into positively charged TMC brings forward different nanocomposite morphologies and properties than CMC as a carrier material. Furthermore, uptake of fluorescein isothiocyanate (FITC) labeled POM–CMC and POM–TMC by HeLa cells was monitored, and the influence of chlorpromazine (CP) as inhibitor of the clathrin mediated pathway revealed different cellular uptake behavior of composites and pristine carriers. TMC/$\{CoW_{11}TiO_{40}\}$ nanocomposites are taken up by HeLa cells after short incubation times around 30 min at low concentrations. The anticancer activity of pristine $\{CoW_{11}TiO_{40}\}$ and its TMC-nanocomposites was investigated *in vitro* with MTT assays and compared to a reference POM.

1. Introduction

Drug delivery techniques with abundant, biodegradable and biocompatible polymers are important for efficient therapeutic approaches. Among these carrier matrices, chitosan can be widely functionalized (Andrade et al., 2011; Sonia & Sharma, 2011) for optimal polymer–drug interactions and release properties (Gaspar, Sousa, Queiroz, & Correia, 2011; Janes, Fresneau, Marazuela, Fabra, & Alonso, 2001; Pahwa et al., 2012; van der Lubben, Verhoef, Borchard, & Junginger, 2001). Controlled release of the encapsulated drugs from chitosan derivatives is facilitated through their degradation by ubiquitous enzymes, e.g. chitosanase and lysozyme (Khor, 2001; Nam et al., 2009; Peng, Tseng, Ho, Wei, Liao, & Sung, 2011). Tailored chitosan derivatives open up the way to safe delivery of new drug prototypes, such as the flexible and growing family of transition metal oxide clusters (preferably of W, Mo and V) (Borras-Almenar, Coronado, Müller, & Pope, 2004; Hill, 1998; Miras et al., 2010) known as polyoxometalates (POMs). Over the past decades, the antiviral, antibacterial and anticancer properties of POMs have been extensively reported on (Fluetsch, Schroeder, Gruetter, & Patzke, 2011; Hasenknopf, 2005; Judd et al., 2001; Menon et al., 2011; Pope & Mueller, 1994; Rhule, Hill, & Judd, 1998; Shigeta, Mori, Yamase, Yamamoto, & Yamamoto, 2006). In sharp contrast to their high bio-medical potential that resulted, for example, in a first round of clinical tests against HIV (Moskovitz, 1988), the biochemical pathways (Hungerford, Suhling, & Green, 2008; Ni et al., 1996; Prudent et al., 2008; Zhang et al., 2007) of POMs remain widely unexplored, because their direct monitoring in cells is a considerable challenge. As manifold phenomenological studies on bio-active POMs and composites thereof (Menon et al., 2011) continue to appear (Fluetsch, Schroeder, Gruetter, & Patzke, 2011; Hasenknopf, 2005; Judd et al., 2001; Menon et al., 2011; Pope & Mueller, 1994; Rhule, Hill, & Judd, 1998; Shigeta et al., 2006), while the according pharmaceutical applications still remain to be developed, fundamental studies into the metabolic behavior of POMs are now required. Additionally, the potential adverse effects of POMs, such as cytotoxicity, need to be reduced for their further exploration as interesting candidates for tuneable and low-cost drugs, e.g. through encapsulation techniques (Han et al., 2011; Meissner et al., 2006). Recently, we have established a new chitosan-based drug carrier approach for POMs that addresses the issues of both POM cytotoxicity and cellular tracking in a dual manner (Geisberger, Paulus, Carraro, Bonchio, & Patzke, 2011). POM nanocomposites with carboxymethyl chitosan (CMC) (Chen & Park, 2003; Jeong et al., 2010; Shi, Du, Yang, Zhang, & Sun, 2006) were formed via a gelation approach and have been proven non-cytotoxic. Moreover, their fluorescent labeling permitted the first precise localization of intact POM composites within HeLa cells,

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where they quickly assemble in the perinuclear region (Geisberger, Paulus, Gyenge, Maake, & Patzke, 2011). As POMs have been identified as potent enhancing agents for antiviral (Shigeta et al., 1997) and antibacterial drugs (Inoue et al., 2006), the growing family of chitosan derivatives offer new opportunities for targeted delivery strategies.

In this study, we introduce a new nanocomposite type of POMs with trimethyl chitosan (TMC) (Mourya & Inamdar, 2009). The positive charge of TMC is favorable for high solubility at neutral and slightly basic pH values and furthermore permits a direct electrostatic encapsulation of highly negatively charged POM polyanions. This is an advantage over the negatively charged CMC polymer matrix which requires an additional $CaCl_2$-mediated gelation step to incorporate POMs. Given that the surface charge of nanoparticles exerts a tremendous effect on their uptake properties (Huang, Fong, Khor, & Lim, 2005; Mailaender & Landfester, 2009; Verma & Stellacci, 2010) and that positively nanoparticular drug carriers frequently display superior uptake properties (Harush-Frenkel, Debotton, Benita, & Altschuler, 2007) we here explore the multiple functions of TMC as a stabilizer, shuttle and charge compensating carrier for POMs.

TMC keeps attracting considerable research interest for a wide spectrum of drug delivery applications encompassing vaccines, various drugs, e.g. insulin (Mi et al., 2008) or desmopressin (Polnok, Verhoef, Borchard, Sarisuta, & Junginger, 2004), small molecules and DNA (Mourya & Inamdar, 2009). TMC is suitable for opening tight junctions (van der Merwe, Verhoef, Verheijden, Kotzé, & Junginger, 2004) and thus facilitates drug delivery in physiological media (Mourya & Inamdar, 2009) or brain drug delivery with low toxicity (Wang et al., 2010). Additionally, TMC opens up manifold chemical routes toward N-alkyl and N-aryl TMCs with individual bio-active profiles, such as antibacterial activity (Jia, Shen, & Xu, 2001). Currently, such compounds are further subjected to grafting processes with other polymers (e.g. PEG) (Liang, Sun, Duan, & Cheng, 2012), thereby paving the way to a new arsenal of copolymeric drug delivery vehicles, and TMC-based crosslinked polymers are used in various nasal vaccination strategies (Slütter & Jiskoot, 2010). The internalization pathways of chitosan-based nanoscale drug carriers often follow diverse endocytosis mechanisms (Park et al., 2010). As a consequence, non-toxic TMC-based polymer shuttles are interesting candidates for the controlled delivery of POMs in order to obtain the long sought-after insight into their behavior under physiological conditions that is still missing for their pharmacological evaluation. Vice versa, POMs serve as model compounds for the interaction of TMC matrices with negatively charged guest molecules.

Inspired by reports on successful anticancer drug delivery with TMC (Liu et al., 2010) and its promising biocompatibility and antitumoral carrier properties *in vivo* (Guan et al., 2012), we studied TMC nanocomposites with $K_6H_2[CoW_{11}TiO_{40}]\cdot 13H_2O$ (henceforth abbreviated as $\{CoW_{11}TiO_{40}\}$) as a representative POM with proven biological and antitumoral activity (Mueller et al., 2006; Wang, Liu, & Pope, 2003; Yang, He, Wang, Li, & Liu, 2004). $\{CoW_{11}TiO_{40}\}$ is furthermore suitable for anticancer composite formation with liposome (Wang, Li, Liu, & Pope, 2005; Yang et al., 2004) and starch (Wang et al., 2003; Zhai, Li, Zhang, Wang, & Li, 2008) as carriers. In the following, we compare the influence of CMC and TMC polymer matrices on the particle size, morphology and surface charge of the resulting hybrid nanocomposites. The influence of the different polymer charges on the uptake efficiency and mechanisms of their respective POM composites is investigated. Furthermore, cellular uptake of FITC-labeled $\{CoW_{11}TiO_{40}\}$–TMC nanocomposites is monitored and the anticancer activity of the new nanomaterials is compared to reference POM–TMC composites.

2. Experimental

2.1. Materials

Chitosan (LMW, 20 kDa, degree of deacetylation > 85%) was purchased from Sigma–Aldrich. All other used reagents were purchased from Sigma–Aldrich or Acros as ACS reagents and used as received.

2.2. Synthesis of POMs

$K_7H[Nb_6O_{19}]$, α_2-$K_{10}[P_2W_{17}O_{61}]\cdot 20H_2O$ and $K_{14}[Na(H_2O)P_5W_{30}O_{110}]\cdot 31H_2O$ were synthesized according to refs. (Alizadeh, Harmalker, Jeannin, Martinfrere, & Pope, 1985; Dickman, Gama, Kim, & Pope, 1996; Edlund, Saxton, Lyon, & Finke, 1988; Ginsberg, 1990).

α_2-$K_7[P_2W_{17}(NbO_2)O_{61}]\cdot 13H_2O$ (Judd et al., 2001): $K_7H[Nb_6O_{19}]$ (55 mg, 0.475 mmol) was dissolved in an aqueous H_2O_2 solution (7.00 mL, 1.5%). HCl (0.20 mL, 4 M) and α_2-$K_{10}[P_2W_{17}O_{61}]\cdot 20H_2O$ (0.66 g, 0.145 mmol) in H_2O_2 (20 mL, 1.5%) were added. HCl (4 M) was added to adjust the pH to 1.1 and KCl (1.0 g, 12.4 mmol) was added. The volume of the solution was reduced in a stream of nitrogen for 12 h. Yellow crystals were formed after the solution was stored for 24 h at 4 °C. The crystals were collected by filtration. Yield: 80 mg (13%).

$K_6H_2[CoW_{11}TiO_{40}]\cdot 13H_2O$ (Chen and Liu, 1997): $Na_2WO_4\cdot 2H_2O$ (1.8256 g, 5.5 mmol) was dissolved in water (10 mL). The pH was adjusted to 6.31 with glacial acetic acid and a solution of $Co(CH_3COO)_2\cdot 4\,H_2O$ (0.1228 g, 0.52 mmol) in water (1 mL) was added dropwise. Afterwards, $TiOSO_4$ (0.16 g, 1.0 mmol) dissolved in 0.1 M H_2SO_4 (1 mL) was added dropwise. The mixture was heated to reflux for 1 h and was then allowed to cool to room temperature. KCl (0.6 g) was added in small portions until no further precipitation was observed. The precipitate was collected by filtration and recrystallized twice from hot water. The product was obtained as blue crystals. Yield: 0.25 g (15%).

2.3. Synthesis and FITC labeling of carboxymethyl chitosan (CMC)

CMC with a degree of substitution of 1.4 per sugar unit and a molecular weight of 20 kDa was prepared as described in ref. (Geisberger, Paulus, Carraro, et al., 2011).

CMC: NaOH (6.75 g) was dissolved in a mixture of deionized water and isopropanol (1:4, 50 mL). Chitosan (5 g) was added and alkalized in this mixture at 50 °C for 1 h. Monochloroacetic acid (7.5 g) was dissolved in isopropanol (10 mL) and slowly added to the reaction mixture over 30 min at 50 °C. The reaction was quenched after 4 h of stirring at 50 °C by addition of ethanol (70%, 100 mL) to the reaction mixture. After collection by filtration the product was extensively washed with 70–94% ethanol to remove residual amounts of salt and water, dialyzed for two days against distilled water and dried by lyophilization. Yield: 10.75 g, white solid.

FITC–CMC: CMC (30 mg) was dissolved in 3 mL distilled H_2O, and a solution of FITC (3 mg) in dry MeOH (4.5 mL) was added, yielding a fluorescent orange mixture which was stirred for 4 h in the dark at room temperature. Afterwards, the solvent was reduced to 1 mL under vacuum and an orange solid precipitated upon addition of ethanol. The solid was washed thoroughly with ethanol until the washing solution showed no more fluorescence. The product was dried under high vacuum and stored in the dark at 4 °C.

2.4. Synthesis and FITC labeling of trimethyl chitosan (TMC)

O-methylated trimethyl chitosan with a degree of quaternization of 20% and a molecular weight of 20 kDa was prepared as described in ref. (Verheul et al., 2008). Chitosan (0.50 g) and sodium