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Effect of Microformulation on the Bioactivity of an Anthocyanin-rich Bilberry Pomace Extract (*Vaccinium myrtillus* L.) in Vitro

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Supporting Information

ABSTRACT: In cell culture were compared the different release rates of anthocyanins from a bilberry pomace extract encapsulated either in food grade whey protein-based matrix capsules (WPC) or in pectin amid-based hollow spherical capsules (PHS). The impact of the formulations on typical anthocyanin-associated biological end points such as inhibition of the epidermal growth factor receptor (EGFR) and suppression of cell growth in HT29 colon carcinoma cells was assessed. The purpose was to find whether the release rates are sufficient to maintain biological activity and whether encapsulation affected EGFR inhibitory and growth suppressive properties of the extract. Even though anthocyanin release from extract-loaded capsules was proven under cell culture conditions, the inhibitory potential toward the EGFR was diminished. However, nonencapsulated extract as well as both extract-loaded encapsulation systems diminished the growth of HT29 cells to a comparable extent. The loss of EGFR inhibitory properties by encapsulation despite anthocyanin release indicates substantial contribution of other further constituents not monitored so far. Taken together, both applied encapsulation strategies allowed anthocyanin release and maintained biological activity with respect to growth inhibitory properties. However, the loss of EGFR inhibitory effects emphasizes the need for biological profiling to estimate process-induced changes of plant constituent's beneficial potencies.

KEYWORDS: *bilberry pomace extract, epidermal growth factor receptor, human colon carcinoma cells, pectin, whey protein*

INTRODUCTION

A diet high in fruits and vegetables has been associated with decreased risk of developing colorectal cancer.[1,2] In this regard especially anthocyanins, colorants in red and blue fruits, are assumed to possess preventive potential.[3−5] Juice and extracts of bilberries (*Vaccinium myrtillus* L.) provide a broad spectrum of anthocyanins, based on the aglycons cyanidin (cy), delphinidin (del), petunidin (pt), malvidin (mv), and peonidin (peo).[6] In vitro as well as in vivo data reported anti-inflammatory, antioxidant, and chemopreventive effects for these glycosides.[3−5] Several members of the human receptor tyrosine kinase (RTK) family have been shown to be inhibited by constituents of extracts rich in anthocyanins.[7] This family of cell surface receptors is thought to play an important role in cancer development and progression.[8−11] In particular, the epidermal growth factor receptor (EGFR), a member of the ErbB subfamily, was shown to be either overexpressed or mutated in diverse tumors.[12−14] Inhibition of the EGFR activity by chemotherapeutics or natural products results in deactivation of downstream mitogenic signal elements, for example, the MAPK or PI3K/AKT pathway, and has been associated with the suppression of tumor growth.[15−17]

Stabilized in compartments of plant cells, anthocyanins are rather unstable in solution, particularly in neutral environments, for example, in food or in the human digestive tract.[18] The bioactivity of anthocyanins is strongly linked to their pharmacokinetic character (site and rate of absorption, metabolic transformation and excretion).[19] Formulation of bioactive food compounds provides a range of beneficial effects. Encapsulation allows masking of unpleasant flavors and odors and offers barriers between bioactive material and other food components to protect unstable compounds from fast degradation or undesired interactions.[20,21] Furthermore, rate and time of release can be controlled to determine the site of action in the human gastrointestinal tract (GIT). Triggers used for prompting the release of encapsulated components could be enzymatic activity, osmotic force, time, or changes of temperature and pH.[22] Major formulation systems applied to oral administration are emulsions,[23] liposomes,[24] hollow sphere capsules,[25] cyclodextrins,[26,27] and matricial particles.[28,29] Present microencapsulation systems are used for lipophilic compounds (e.g., fatty acids, carotenoids, or phytosterols), water-soluble vitamins, minerals, enzymes, and microorganisms (e.g., yeasts or probiotics).[21,30]

In the present study we investigated whether the bioactivity of an anthocyanin-rich bilberry pomace extract is affected by encapsulation into (a) food grade whey protein based matrix capsules (WPC) or (b) pectin amid-based hollow spherical capsules (PHS). With HT29 cells as a model cell line originating from the human GIT, we determined the release of bioactive constituents from the two encapsulation systems in

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the cell culture medium exemplarily for anthocyanins in relation to the impact of encapsulation on well-characterized biological end points such as growth inhibition and the suppression of the EGFR. The aim of the study was to find whether the release rates from the applied encapsulation systems are sufficient to maintain biological activity or whether encapsulation affects the bioactivity profile of the extract.

MATERIALS AND METHODS

Chemicals. All chemicals and solvents were of analytical grade or complied with the standards needed for cell culture experiments. Reference cyanidin-3-*O*-glucoside chloride (cy-3-glc) and delphinidin-3,5-di-*O*-glucoside chloride (del-3,5-diglc) were purchased from Extrasynthese (Genay Cedex, France). HT29 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Amidated pectin (Pectin amid AU-L 027/09) was provided by Herbstreith & Fox KG, Neuenburg/Württ., Germany. $CaCl_2 \cdot 2H_2O$ and anhydrous glycerol (≥99%) were purchased from Merck Chemicals, Darmstadt, Germany. Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, trypsin, and fetal calf serum (FCS) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Cell culture flasks, Petri dishes, and centrifuge and microcentrifuge tubes were purchased from Sarstedt (Nuembrecht, Germany). Catalase was ordered from Carl Roth (Karlsruhe, Germany). Monoclonal primary and secondary antibodies were purchased from Santa Cruz (Heidelberg, Germany), and nitrocellulose (0.2 μm) was from Pall Corp. (Port Washington, NY, USA).

Bilberry Pomace Extract (BE). BE (600761 bilberry extract; Symrise GmbH & Co. KG, Holzminden, Germany) was produced from European bilberry pomace (*V. myrtillus* L.) by extraction with methanol, filtration, evaporation, and lyophilization. The constituents were as follows (Symrise, datasheet): total polyphenol content (Folin−Ciocalteu), 46% wt; dietary fibers, 28.8% wt, carbohydrates (glucose and fructose), 1.55% wt. The anthocyanin content was determined as 27.6% wt within this study. The extract was characterized with respect to polyphenol composition by Juadjur et al.[31] and was applied earlier in cell culture studies.[32] It was stored dry and cold at −80 °C.

Extract Encapsulation. Whey protein based matrix capsules (WPC) were prepared and characterized as described previously.[33] The average particle diameter ($x_{50,3}$) of WPC was 412 μm. Amidated pectin hollow spheres (PHC) were prepared by extrusion, using a simple one-step process.[34,35] An aqueous amidated pectin solution of 0.5% wt was used as polymer solution. For the preparation of the cross-linking solution, 3.0% wt $CaCl_2 \cdot 2H_2O$ salt and 2% wt BE were dissolved in 100 mL of double-distilled water. This solution was stirred for at least 10 min to dissolve the extract to the greatest possible extent. Then this mixture was centrifuged at 5000*g* for 30 min to remove the nonsoluble extract particles. Afterward, the supernatant solution was diluted with an equal volume of anhydrous glycerol, which was added to improve the mechanical properties as well as the density for facilitating the dropping-in. The cross-linking solution contained 50% vol glycerol and a resultant concentration of 1.5% wt $CaCl_2$ as well as 1.0% wt BE. In the case of the blank capsules, the cross-linking solution was prepared in the same manner, only without addition of the BE.

In the capsule production process, first the cross-linking solution was added dropwise by means of a high-precision metering syringe into the aqueous 0.5% wt pectin solution. A capsule membrane is formed instantly around each droplet once the two liquids came into contact.[36] To avoid aggregation of the prepared capsules, the pectin solution was constantly moved by using a magnetic stirrer. A dropping height of 4 cm was used to ensure that nearly spherical capsules were formed. After a gelation time of 30 s, the PHC were separated, filtered, washed with double-distilled water, and transferred into a 1.0% wt $CaCl_2 \cdot 2H_2O$ solution, also containing 1% wt BE with the aim of completing the polymerization process and inhibiting the anthocyanin diffusion during the transportation and storage period (approximately 1 week). Average diameter of the particles was 1−3 mm.

Cell Culture. The human colon carcinoma cell line HT29 was cultivated in DMEM (4500 mg/L glucose, without sodium pyruvate) supplemented with 10% vol FCS and 1% vol penicillin/streptomycin for not more than 30 passages. Cell culture medium was changed on a regular basis (2−3 days), and cells were passaged at <80% confluence. Cultivation and incubation of the cells were carried out in a humidified incubator (37 °C, 5% CO_2).

Anthocyanin Quantitation by HPLC. HT29 cells (1,000,000) were seeded in culture plates (d = 10 cm) 72 h prior to treatment to obtain a 70−80% confluence. After removal of the culture medium, substance and particle incubations (maximum anthocyanin release from capsule system was identified beforehand), incubation was carried out with 200 μg/mL BE or the corresponding amount of capsules, respectively. Because the anthocyanin concentration of the extract was 27.6% wt, 200 μg/mL extract corresponds to 55.2 μg/mL (113.9 μM) anthocyanins, calculated as cyanidin-3-glucoside chloride (MW 484.8 g/mol). Capsule extract content was determined prior to release studies by HPLC. Analysis of the anthocyanin content was performed in serum-free DMEM and as a control in acified DMEM (5% vol formic acid), both containing 100 U/mL catalase. Catalase was used in all experiments to diminish hydrogen peroxide formation as reported previously.[37] Samples (95 μL (cell-free experiment) and 85 μL (cell-based experiment), respectively) were withdrawn after 0, 5, 10, 15, 30, 45, 60, and 120 min, respectively, subsequently acidified with formic acid (10 μL for the cell-based experiment), and supplemented with del-3,5-diglc solution (5 μL, 1 mM) as an internal standard. After centrifugation for 2 min (20000*g*), 10 μL of the solution was injected on an Agilent Technologies 1200 series HPLC system (Santa Clara, CA, USA) equipped with a diode array detector (DAD). The separation was performed on a C18 column (Phenomenex Luna 3 μm, 250 × 4.6 mm) at 30 °C. Gradient chromatography was used with an H_2O/acetonitrile gradient. Solvent A was H_2O/acetonitrile/formic acid, 87:3:10 (v/v/v), and solvent B was H_2O/acetonitrile/formic acid, 40:50:10 (v/v/v). The gradient was as follows: 0 min, 2% B; 20 min, 14% B; 25 min, 14% B; 40 min, 18% B; 45 min, 18% B; 70 min, 90% B; 80 min, 2% B; 90 min, 20% B. Anthocyanins were detected at 520 nm. Quantitation was carried out using peak areas from external calibration with cy-3-glc while standardizing on del-3,5-diglc. The limit of detection (LOD, 0.224 μg/mL) and limit of quantitation (LOQ, 0.787 μg/mL) were calculated for cy-3-glc. The areas under the curve (AUCs) were calculated as percent of AUC from nonencapsulated extract (T/C).

Western Blot Analysis. HT29 cells (1,000,000) were seeded in culture plates (d = 10 cm) 72 h prior to treatment to obtain a 70−80% confluence. Twenty-four hours before treatment, the initial medium was replaced by DMEM containing 1% vol FCS. Substance and particle incubations were performed in serum-free DMEM containing 100 U/mL catalase for 45 min, and 1, 2, and 3 h, respectively. In all cell culture experiments for analysis of EGFR phosphorylation with encapsulated material, the applied amount of capsule material was held constant in every sample by incubation with the respective amount of BE-loaded particles (calculated according to the results on anthocyanin content) plus nonloaded encapsulation material to reach the amount of encapsulation material in the highest applied BE-loaded preparation. Because initial experiments did not indicate a significant difference between medium control and the unloaded particle material on the status of EGFR phosphorylation, nonloaded encapsulation material was used as control. Fifteen minutes prior to the end of incubation, cells were treated with human epidermal growth factor (EGF, 100 ng/mL). Cells then were rinsed twice with phosphate-buffered saline (PBS) and homogenized using 200 μL/cell culture plate (d = 10 cm) of RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% vol Igepal, 1 mM PMSF, 1 mM sodium orthovanadate, and 2% vol protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)) at 4 °C. Solid cell components were removed by centrifugation for 10 min (20000*g*, 4 °C). Afterward, the protein concentration of the supernatants was determined according to the method of Bradford.[38]

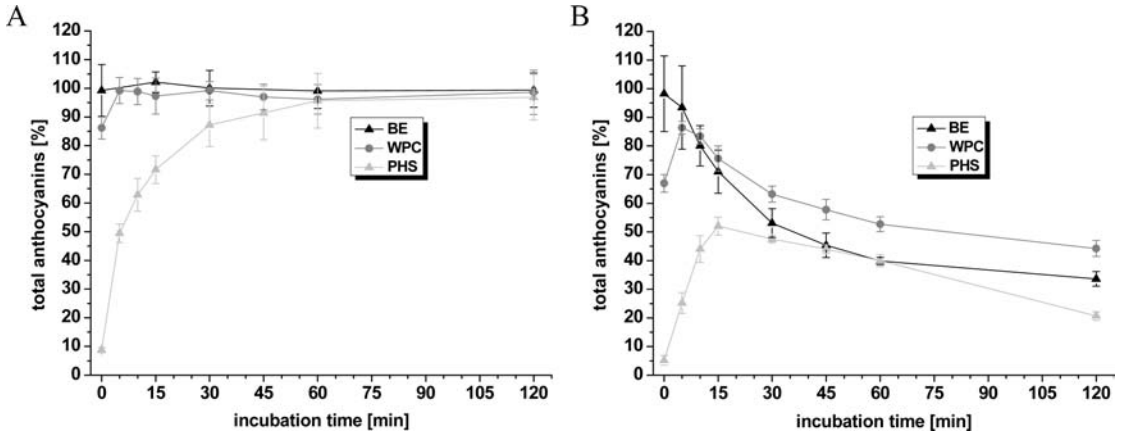

Figure 1. Anthocyanin content in the cell culture medium after application of BE, WPC, and PHS, respectively: (A) cell-free at pH 2; (B) in the supernatant of cultured HT29 cells at pH 7.8. BE was dissolved in DMEM. Samples were taken at the indicated time points, and the anthocyanin concentration was measured by HPLC-DAD. Quantitation was carried out using peak areas from external calibration with cy-3-glc while standardizing on del-3,5-diglc. Values, which are expressed as the percentage of nonencapsulated BE anthocyanins, are the mean ± SD of three independent experiments.

Table 1. Parameters of Anthocyanin Release from Different Capsule Systems[a]

		c_{max}		AUC	
preparation	t_{max} (min)	μM	%	mM × min	%
BE	0.00	120.3 ± 16.0	98.2 ± 13.1	7.10 ± 0.53	100.0 ± 0
WPC	5.00	105.8 ± 2.8	86.4 ± 2.3	8.43 ± 0.27	119.2 ± 10.2
PHS	15.00	63.6 ± 3.9	52.0 ± 3.2	5.35 ± 0.29	75.4 ± 3.1

[a] t_{max}, time of maximum anthocyanin concentration; c_{max}, maximum anthocyanin concentration; AUC, area under the curve.

Sixty micrograms of total protein of each sample was separated by SDS-PAGE (7% polyacrylamide gel) and subsequently blotted on a nitrocellulose membrane (0.2 μm). Total EGFR and phospho-EGFR (Tyr 1173) (both diluted 1:1000) were detected using appropriate primary monoclonal mouse/goat antibodies. Secondary peroxidase-conjugated antibodies against mouse and goat (both diluted 1:2000), respectively, were used to obtain chemiluminescence signals along with standard ECL (Perkin-Elmer, Waltham, MA, USA). Signals were captured by a CCD-imager (Fujifilm LAS 4000, Tokyo, Japan) and quantified using the analysis software Multi Gauge 3.2 (Fujifilm). Protein levels were calculated as test over control (T/C) of the respective light units and are given in percent.

Cell Growth Inhibition. HT29 cells (5000 cells) were seeded in culture plates (d = 6 cm) 24 h prior to treatment to obtain a 10% confluence. Substance and particle incubations were performed in DMEM containing serum and 100 U/mL catalase for 72 h. Afterward, cells were rinsed twice with PBS (37 °C) and subsequently treated with trypsin (0.5 mg/mL trypsin, 0.25 mg/mL EDTA) for 3 min. Trypsin activity was stopped by adding serum-containing medium (10% vol FCS). Living cells were counted after staining with trypan blue. Cellular growth was calculated as test over control (T/C) of living cell numbers and is given in percent.

Statistical Analysis. Data from in vitro experiments were analyzed by a two-tailed, homoscedastic t test (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

RESULTS AND DISCUSSION

Anthocyanins Are Stabilized by Microencapsulation. Anthocyanin release from both applied microencapsulation systems was determined in cell culture medium. Fifteen different anthocyanins composed from the five aglycons, del, cy, pt, mv, and peo, and the three glycons, glucose (glc), galactose (gal), and arabinose (arab), were quantified as cy-3-glc equivalents by HPLC-DAD. Most abundant in the BE without degradation were del-glycosides (34.3% wt) followed by cy-glycosides (27.6% wt). Pt-, mv-, and peo-glycosides were represented with 15.6, 13.7, and 8.7% wt, respectively (see the Supporting Information, Figure 1).

Complete anthocyanin release compared to the non-encapsulated extract without detectable degradation was observed in acidified medium (pH 2) without cells (Figure 1A). Anthocyanins of BE-loaded WPC were found to be released completely within 5 min (Figure 1A). Already at the first point of measurement, directly after suspending the particles in the culture medium, 86 ± 4% of total anthocyanins were released. In contrast, release from BE-loaded PHS took up to 1 h (Figure 1A). In the presence of HT29 cells at pH 7.8 the total anthocyanin concentration of BE decreased within 2 h to 29 ± 2% (Figure 1B). The rate of decrease of the anthocyanin content appeared to be substantially affected by their respective aglycon. Whereas cy-, pt-, ma-, and peo-glycosides were rather stable, del-glycosides disappeared completely within 30 min (data not shown). Thus, the initial and fast decrease in total anthocyanin concentration was mostly caused by the quick degradation of del-glycosides, which in fact represented the predominant proportion. Anthocyanins from BE-loaded WPC exhibited a burst release in the first 5 min, which was subsequently overlapped by anthocyanin degradation. In contrast, anthocyanins from PHS were released more slowly; hence, release and degradation of the anthocyanins overlapped during a longer time period. As a consequence, the maximum anthocyanin concentration was lower compared to the application of nonencapsulated extract. After 2 h of incubation, the AUC of total anthocyanins of BE-loaded WPC was significantly higher (120%, Table 1) than that from non-encapsulated extract, whereas the AUC resulting from BE-loaded PHS was significantly lower (63.6%, Table 1).

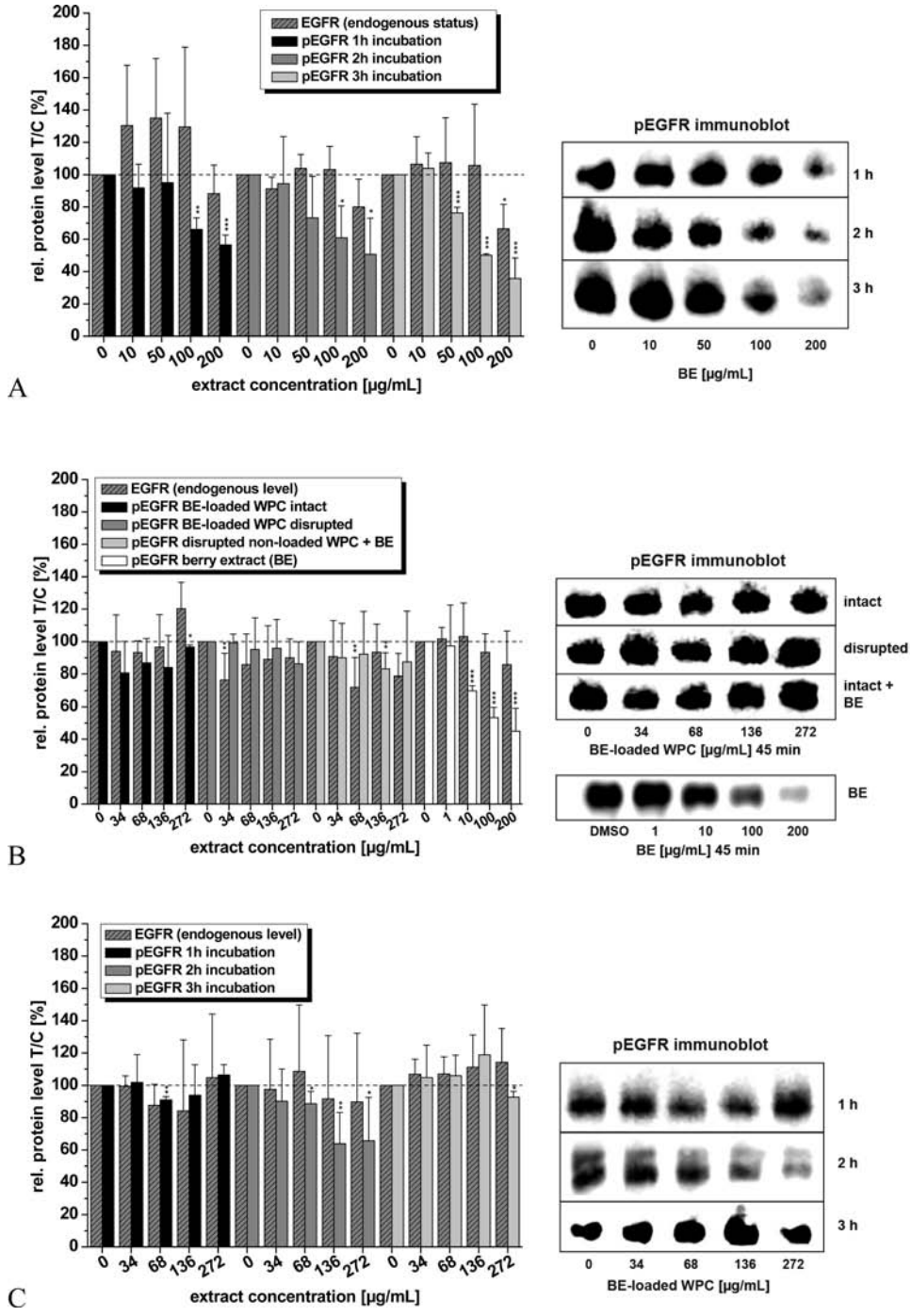

Figure 2. Effect of bilberry extract and BE-loaded WPC on the phosphorylation of the EGFR in HT29 cells. (A) Cells were exposed to the extract for 1, 2, and 3 h, respectively. (B) Cells were exposed to intact and disrupted particles loaded with BE in suspension for 45 min, stimulated with EGF (100 ng/mL). Furthermore, cells were treated with unloaded particles in co-incubation with nonencapsulated BE. Values, which are expressed as the percentage of unloaded control WPC, are the mean ± SD of three independent experiments. Statistical significance was calculated in relation to the negative control (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

The results indicate that not only was anthocyanin liberation affected by encapsulation but also their stability in cell culture medium (Figure 1). The pH value of the cell culture medium was not affected by capsule application (data not shown); hence, the known pH dependency of anthocyanin stability should not play a role for the observed effect. Taken together, application of BE-loaded WPC leads to a lower peak concentration but a higher overall anthocyanin AUC in comparison to the same amount of nonencapsulated BE (Figure 1B). Anthocyanins were already reported to show some affinity to whey protein. In whey protein-stabilized emulsions berry anthocyanins were found to be associated with whey proteins to about 20%.[39] This effect might contribute to the observed stabilizing effect of the WPC preparation. The low overall anthocyanin AUC obtained from PHS may be referred to the vast overlap of slow release and fast degradation of anthocyanins in this system.

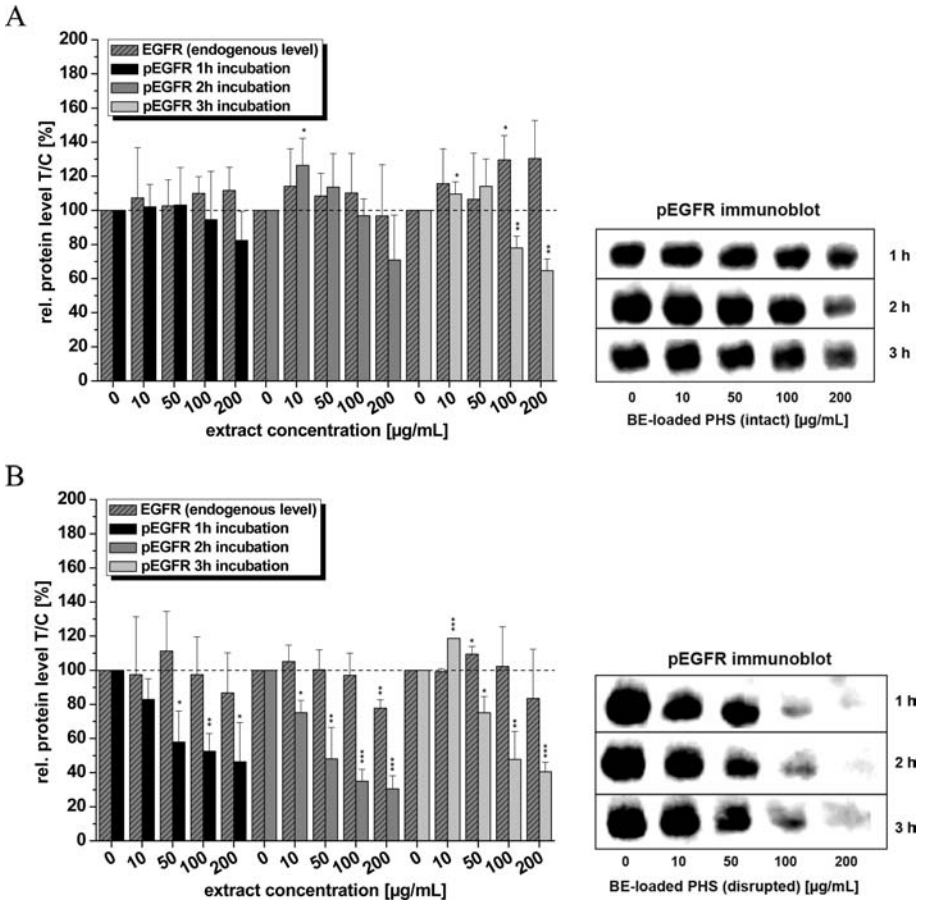

Figure 3. Effect of bilberry extract loaded PHS on the phosphorylation of the EGFR in HT29 cells. Cells were exposed to the (A) intact and (B) disrupted spheres in suspension for 1, 2, and 3 h, respectively, stimulated with EGF (100 ng/mL). Values, which are expressed as the percentage of unloaded control PHS, are the mean ± SD of at least three independent experiments. Statistical significance was calculated in relation to negative control (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.005$).

Encapsulation Modulates BE's Bioactivity in Terms of EGFR Activity. Polyphenol-rich plant extracts and various flavonoids have already been shown to inhibit RTK activity in vitro.[16,40,41] Especially extracts rich in anthocyanins and the respective sugar free counterparts, the anthocyanidins, are known to act as potent inhibitors of proliferation-associated cellular signaling cascades.[15,17,42] This pharmacologically relevant attribute of these substances may contribute to their chemopreventive potential in vivo.[43,44] Therefore, the inhibitory effects on EGFR activity and tumor-cell proliferation were used as parameters to estimate the influence of encapsulation on the bioactivity of the anthocyanin-rich BE. Western blot analysis was used to measure EGF-induced receptor phosphorylation, which reflects kinase activity. The treatment with nonencapsulated BE for 1, 2, and 3 h resulted in a concentration-dependent suppression of EGFR phosphorylation (Figure 2A). In all tested incubation periods receptor phosphorylation was diminished significantly to at least 66 ± 7% (T/C) already by treatment with 100 µg/mL BE. Application of 200 µg/mL BE resulted in activities of approximately 50% (T/C) after 1 and 2 h of incubation, respectively, and 36 ± 13% (T/C) after 3 h. The endogenous level of the receptor was not influenced significantly by BE treatment except after 3 h of incubation. Here the total amount of receptor was decreased to 66 ± 15% (T/C) at the highest concentration of 200 µg/mL, indicating effects on the total receptor status.

To investigate the effect of extract-loaded WPC on EGFR activity, HT29 cells were incubated with intact capsules. Due to the expected direct release of the BE constituents and aiming to minimize substance degradation after release, the subsequent cell culture experiments were performed with an incubation time of 45 min, a time span well established for the assessment of modulation of EGFR phosphorylation by natural compounds.[45−47] Capsule treatment was performed in concentrations standardized with respect to BE content, determined prior to the cell culture experiments by HPLC-DAD after complete anthocyanin release. BE content was calculated with respect to the anthocyanin concentration (referring to 27.6% anthocyanins in BE). Furthermore, particles were disrupted by ultraturrax treatment before incubation to release the extract constituents instantaneously. Whereas incubation of HT29 cells for 45 min with nonencapsulated BE led to a significant suppression of EGFR activity (Figure 2B), treatment neither with homogenized nor with intact WPC showed substantial EGFR inhibitory properties. To address the question of whether the protein material of the WPC preparation might be of relevance for the loss in EGFR inhibitory properties, HT29 cells were incubated with nonencapsulated extract in combination with disrupted nonloaded WPC material (Figure 2B).

Because no effect on EGFR activity was determined due to capsule treatment for 45 min, HT29 cells were incubated with intact BE-loaded WPC for 1, 2, and 3 h, respectively, to address

the question of whether the suppression of EGFR phosphorylation is shifted to a later time point. No significant effect on EGFR phosphorylation was detectable by incubation with the BE-loaded WPC after 1 and 3 h of incubation, respectively (Figure 2C). Merely after 2 h was a slight but significant decrease in receptor phosphorylation to about 64 ± 19% (T/C) observed. The endogenous level of the receptor was not significantly influenced by capsule incubation (Figure 2C). These results indicated a slight time shift for BE-loaded WPC, but with clearly diminished effectiveness compared to nonencapsulated BE with respect to extent and duration of the effect. However, the release of anthocyanins (see Figure 1B) was not reflected by the impact on the phosphorylation status of the EGFR (Figure 2C).

To determine the effect of BE release from PHS on EGFR phosphorylation, HT29 cells were incubated with intact BE-loaded PHS for 1, 2, and 3 h, respectively. Unloaded PHS were used as control. Incubation with extract-loaded capsules resulted in a concentration-dependent as well as time-dependent suppression of EGFR activity (Figure 3A), with a delayed onset and reduced effectiveness compared to the nonencapsulated BE (compare Figure 2A). The maximum of inhibitory effect on receptor phosphorylation (to 65 ± 7%) was reached after 3 h of incubation. To differentiate between capsule matrix effects and impact of delayed release, HT29 cells were also incubated with BE-loaded PHS, which were disrupted by ultraturrax treatment prior to cell incubation (Figure 3B). In contrast to disrupted WPC, suppression of EGFR activity triggered by disrupted PHS was comparable to the nonencapsulated extract (compare Figure 2A), indicating that the capsule material of the PHS per se did not affect the bioactive properties of the extract.

Taken together, encapsulation of BE in WPC resulted in a substantial loss of EGFR inhibiting potential, which did not correlate with anthocyanin release (compare Figures 1B and 2B). In contrast, incubation with intact BE-loaded PHS led to a decrease in EGFR inhibitory properties, in line with the lower AUC (compare Figures 1B and 3A). Disruption of PHS capsules restored the inhibitory potency with respect to EGFR inhibition, which was not achieved by disruption of BE-loaded WPC (Figure 3B). These observations indicate the following: (1) Whereas pectin amid does not interfere with bioactive BE compounds, there seems to be some interaction between these constituents and the WPC protein. HPLC analysis revealed that at least most of the anthocyanins were released and even stabilized in the cell culture medium. (2) Yet unidentified bioactive BE constituents other than anthocyanins might be trapped in the capsule matrix or might be bound to proteins. Potential candidates for bioactive constituents would be proanthocyanidins or quercetin glycosides. Both classes are known to interact with proteins[48,49] and have a proven impact on EGFR activity.[16,50] Furthermore it might be speculated that bioactive compounds, for example, tannins, may be removed during capsule formation. In this formation process, whey proteins have been observed to interact with parts of the BE and form an insoluble precipitate (see Supporting Information, Figure 2). This precipitate is removed by centrifugation prior to heat-induced capsule formation. The sediment (10.4% wt of total protein solution) can be separated further into insoluble BE constituents and tannin−protein complexes (3.75 and 6.65% wt of total protein solution, respectively). Several tannins were described before to inhibit EGFR activity.[16,45,50] Anthocyanin-rich berry extracts are well-known for their EGFR inhibitory properties.[7] However, the contribution of the different polyphenols to the EGFR inhibitory properties of BE has not been elucidated yet. The free aglycons of anthocyanins, the anthocyanidins, bearing vicinal hydroxyl groups at the B-ring represent potent inhibitors of the EGFR,[15,17,47] but are not present as such in the native extract and, moreover, would not be stable at the neutral pH of the cell culture medium.[51] So far, the literature on the effects of anthocyanins on RTKs is scarce. Under cell-free conditions cy-3-gal was found to be inactive toward the receptor tyrosine kinase activity of the EGFR up to 100 μM.[15] Considering the discrepancy in the present study between the release of anthocyanins from the encapsulation systems and the impact on EGFR phosphorylation, we determined the EGFR-inhibitory properties of the effect of cy-3-glc, one of the most abundant anthocyanins in BE. After 45 min of incubation, cy-3-glc did not affect the phosphorylation status of the EGFR up to 200 μM (data not shown), a concentration exceeding the molarity of anthocyanins present in 200 μg/mL BE, which has been shown to induce significant suppression of EGFR phosphorylation. Taken together, the results of the present study indicate so far, at least, a subordinate role of anthocyanins for effects on EGFR phosphorylation.

Effect of Encapsulation on BE's Growth Inhibitory Potential. As a second potential biomarker of the chemopreventive potential of BE, effects on the proliferation of HT29 cells were determined. Anthocyanin-rich plant extracts have been shown previously to inhibit tumor cellular growth.[7,17] Preliminary experiments indicated that the presence of the capsule material interferes with several methods to assess cell proliferation, for example, by enhancing the protein content by WPC matrix in the sulforhodamine B assay (data not shown). Thus, cell number and viability were determined by the trypan blue assay after 72 h of incubation. The nonencapsulated BE inhibited cell growth significantly in concentrations >100 μg/mL. At a concentration of 200 μg/mL, BE diminished the cell number to 72 ± 19% (T/C), an effect that was enhanced at 400 μg/mL to 31 ± 9% of the solvent control (Figure 4). Incubation with BE-loaded WPC and PHS was found to

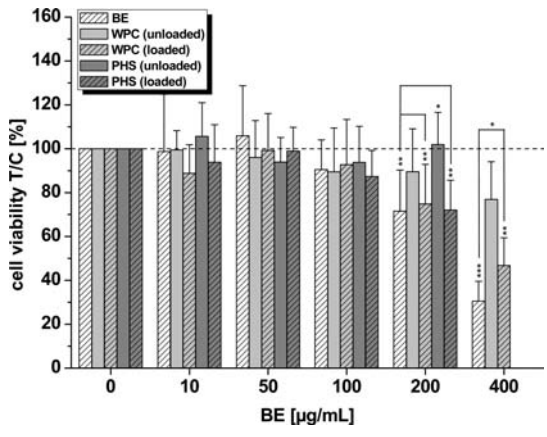

Figure 4. Effect of bilberry extract and bilberry extract loaded capsules on the growth of HT29 cells. Cells were exposed to the extract and intact spheres (unloaded + BE loaded) in suspension for 72 h in the presence of catalase (100 U/mL). Living cells were quantified using trypan blue exclusion assay. Values, which are expressed as the percentage of solvent control, are the mean ± SD of at least three independent experiments. Statistical significance was calculated in relation to negative control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

significantly decrease the cell number at concentrations representing 200 μg/mL BE (75 ± 18% (T/C) for WPC and 72 ± 14% (T/C) for PHS, comparable to the effect of nonencapsulated BE. At 400 μg/mL this effect was even stronger for BE-loaded WPC. In this case, the nonencapsulated BE had a significantly stronger inhibitory potential (plus 16.2% inhibition compared to BE-loaded WPC). BE-loaded PHS could not be included in the testing in that concentration range due to limited material availability. No significant effect on cell growth was observed after incubation with unloaded control WPC and PHS material.

In contrast to the results on EGFR phosphorylation, BE's inhibitory potential with regard to tumor-cell proliferation was altered by encapsulation only at the highest test concentration. Different BE constituents, for example, ellagic acid, anthocyanins, or quercetin, were described before to inhibit tumor cell growth.[45,52−54] Because different pattern of the inhibition of cell growth and the suppression of EGFR activity were found by the encapsulated and free BE, respectively, it might be assumed that the effects on these two biological end points are not directly linked. It is therefore likely that either cellular proliferation of HT29 cells is not exclusively dependent on EGFR/MAPK regulation[17] or, probably due to encapsulation, modulation of EGFR activity occurs in a yet unknown time frame.

Encapsulation of bioactive food compounds is promising with regard to application as food additives and provides a range of utilizations in the new and rising field of functional foods. Especially chemically unstable compounds may be protected in a strong acidic but food grade environment.[55] Furthermore, the site and rate of compound release in the human GIT may be affected by variation of matrix, size, and coating of the particles.[22] Even though release of marker compounds may be monitored by analytical methods, for example, HPLC, the interaction of released compounds, especially of complex plant extracts, has to be determined within biological assays, simulating the physical environment. In particular for anthocyanins, the site of release in the GIT is important, because resorption and stability of these compounds substantially differ between the stomach and small intestine.[56] Anthocyanin liberation in the human stomach provides a higher systemic availability, placing emphasis on their antioxidant character.[57] Release in the small intestine, on the other hand, might give rise to their putative chemopreventive impact on the intestinal epithelium.[43,44]

Taken together, encapsulation of BE was found to modify the release and stability of anthocyanins under cell culture conditions. The EGFR inhibitory potential of the extract was found to be diminished due to encapsulation, whereas the inhibitory effect on cellular proliferation was only marginally affected. Furthermore, anthocyanin release and the monitored biological effects were not correlative, emphasizing the need for further biomarkers and monitoring of additional bioactive constituents to be included in future studies on the impact of encapsulation, especially in the case of complex preparations to provide a comprehensive bioactivity profile.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

BE, bilberry extract; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; WPC, matricial whey protein capsules; PHS, pectin amid hollow spheres; cy, cyanidin; del, delphinidin; AUC, area under the curve

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